# Barnacle settlement behaviour in response to con- and allo-specific cues

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# Abstract

The project aimed to investigate the utility of selected temperate barnacles as a model species for recently-developed laboratory assays, and to progress the understanding of barnacle con- and allo-specific settlement behaviour through laboratory and field experiments, and the partial characterisation of the adult cue.

*Elminius modestus* larval settlement assays using a 24-well plate assay indicated a preferred settlement temperature of 22±1°C, and gregarious settlement was demonstrated significantly at a Settlement Factor (SF) concentration of 10 $\mu$g $ml^{-1}$. Experiments on the effects of ageing revealed a pattern to settlement not previously observed in the larval community, with high settlement when cyprids were young, reduced when mid-aged, and then increasing with increasing age with an accompanying loss of discrimination to settlement cues. It was questioned whether this may be due to the species physiology, or the presence of different phenotypes. Similar experiments with *Semibalanus balanoides* wild cyprids were far from conclusive, though the preferred settlement temperature was 19±1°C. Further laboratory experiments using *S. balanoides* cyprids were not pursued

The active adult protein, known as settlement-inducing protein complex (SIPC), was isolated from *E. modestus.* One subunit was selected for amino acid sequencing, and subsequent gene isolation by molecular methods. Two short amino acid sequences were isolated from the protein; 1) ATPSLPDNI and 2) QYTYEYEAK.

Laboratory experiments investigating settlement behaviour of *E.modestus* and *Balanus amphitrite* larvae to SF, from six species including the conspecific, showed that settlement by each species was significantly different to different concentrations of each SF tested. However, comparisons between different SFs of the same concentration were not significantly different. Results of choice assays, using several species' SFs in a single experiment, again indicated that there was no significant difference in settlement between different species. In field experiments *S. balanoides* settlement preferences were defined as:-

*S. balanoides* > *E. modestus* > (*B. improvisus* = *B. crenatus* = *C. montagui*)

(where '>' indicates a significant preference and '=' indicates no significant difference), while settlement by *E. modestus* cyprids was the same to all SFs.

# Contents

# List of abbreviations

| | |
|---|---|
| A | adenine |
| A | alanine (amino acid) |
| A2M | alpha-2 macroglobulin |
| $A_{260}$ | absorption at 260 nm |
| $A_{280}$ | absorption at 280 nm |
| ANOVA | analysis of variance |
| BA | *Balanus amphitrite* |
| BC | *Balanus crenatus* |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue alkaline phosphatase |
| BCW | barnacle conditioned water |
| BI | *Balanus improvisus* |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair |
| BSA | bovine serum albumin |
| C | cytosine |
| ca. | circa |
| CAPS | n-cyclohexyl-3-aminopropanesulphonic acid |
| cDNA | complimentary deoxyribonucleic acid |
| cm | centimetre |
| CM | *Chthamalus montagui* |
| D | aspartic acid (amino acid) |
| Da | dalton |
| $dH_2O$ | deionised water |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| E | glutamic acid (amino acid) |
| EM | *Elminius modestus* |
| ERMS | European Record of Marine Species |
| et al. | et alia |
| g | gram |
| G | guanine |
| HPLC | high pressure liquid chromatography |
| Hz | Hertz |
| I | isoleucine (amino acid) |
| IBMX | 3-isobutyl-1-methylxanthine |
| IPTG | isopropyl-ß-D-thiogalactopyranoside |
| K | lysine (amino acid) |

| | |
|---|---|
| kDa | kiloDalton |
| km | kilometre |
| L | leucine (amino acid) |
| l | litre |
| LB | Luria-Bertani (media) |
| L: D | light:dark |
| M | methionine (amino acid) |
| M | molar |
| $\mu$g | microgram |
| $\mu$l | microlitre |
| $\mu$m | micrometre |
| m | metre |
| mA | milliampere |
| mg | milligram |
| min | minute |
| ml | millilitre |
| mm | millimetre |
| mM | millimolar |
| mol | mole |
| mRNA | messenger ribonucleic acid |
| mtDNA | mitochondrial deoxyribonucleic acid |
| MW | molecular weight |
| mya | million years ago |
| N | asparagine (amino acid) |
| nm | nanometre |
| P | proline (amino acid) |
| PCR | polymerase chain reaction |
| pH | potential of Hydrogen |
| pmol | picomole |
| PVDF | polyvinylidene fluoride |
| Q | glutamine (amino acid) |
| RACE | rapid amplification of cDNA ends |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| S | serine (amino acid) |
| SB | *Semibalanus balanoides* |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SF | settlement factor |
| SIPC | settlement-inducing protein complex |

| | |
|---|---|
| sp. | species |
| T | thiamine |
| T | threonine (amino acid) |
| TAE | tris/glacial acetic acid/EDTA |
| TBS | tris-buffered saline |
| TBT | tributyltin |
| Tm | melting temperature |
| TotRNA | total ribonucleic acid |
| Tris | tris (hydroxymethyl) aminomethane |
| TTBS | tween tris-buffered saline |
| U | units |
| UK | United Kingdom |
| UV | ultra violet |
| V | valine (amino acid) |
| V | volt |
| Y | tyrosine (amino acid) |
| w/v | weight/volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside |
| º | degrees |
| ºC | degrees Celsius |

# Chapter 1

# An introduction to barnacles and their settlement behaviour

## 1.1 Overview of the Cirripedia

Barnacles, crustaceans of the subclass Cirripedia, with more than one thousand exclusively marine species (Anderson, 1994), may unequivocally be regarded as successful organisms. While larval forms are planktotrophic, the sessile habitats of adults range from the highest intertidal zone to deep-sea locations and additionally include species with commensal, symbiotic and parasitic relationships with other marine organisms. Geographically, they are located from equatorial to polar regions, while fossil records confirm the existence of early forms from the Cambrian period (570-505 million years ago) (Anderson, 1994).

First identified as crustaceans in 1829 (Thompson, 1830), the work by Charles Darwin (1851, 1854), exemplifying "his great talents of descriptive morphology and classification" (Crisp, 1983), has since provided a foundation of knowledge still relevant today. Interest in barnacles has been sustained not only by the prevalence of species, with many easily accessible in the intertidal zone, but also by the fact that certain species are significant fouling organisms with deleterious economic effects. For example, fouling by marine organisms can increase the fuel consumption of an average-sized merchant vessel by more than 25% (Chandrasekaran and Gnanojothi, 1998). Additionally, the use of tributyltin (TBT) compounds as antifouling biocides is to be phased out completely. Although they were effective antifouling agents, they have been used with considerable environmental costs (Alzieu, 1998; Bryan et al., 1986; Langston et al., 1990). The search for alternative effective methods to non-toxic anti-fouling has heightened the need to have a detailed understanding of barnacles and their settlement behaviour.

**Barnacle phylogeny**

Barnacle classification lacks an overall consensus (Schram and Høeg, 1995), but continues to evolve as knowledge of the taxonomy and phylogeny of the species increases. The subclass Cirripedia is divided into four superorders: 1) the Ascothoracica, which contain parasitic forms found on echinoderms and cnidarian corals; 2) the Acrothoracica, which burrow into calcareous substrates; 3) the Thoracica, which is a highly radiated group with many different forms - it includes

the ordinary barnacles found along the sea-shore, as well as symbiotic and some parasitic forms; 4) the Rhizocephala, which are endoparasites with modified form, such that only the larval stages are recognised as that of the Cirripedia (ERMS, 2001).

The scope of this study is limited to a selection of thoracican species, in particular from the Balanomorpha and Figure 1.1 illustrates the taxonomic structure of the living species of the Thoracica. Recent analysis of thoracican evolution (Pérez-Losada et al., 2004), using molecular and morphological evidence, determined that the Iblomorpha were the most primitive thoracicans and the plateless Heteralepadomorpha were the sister group of the Lepadomorpha. Sessilia barnacles were monophyletic and appeared to have evolved from a stalked pedunculate multiplated (+5) scalpelloid-like ancestor. The Balanomorpha are a group of sessile barnacles with bilaterally symmetrical shells and many have a membranous or calcareous base (Barnes, 1989). The group consists of four superfamilies, the Chthamaloidea, Coronuloidea, Tetraclitoidea and Balanoidea (Newman and Ross, 1976 and 1977; Pérez-Losada et al., 2004), while species of a further superfamily, the Pachylasmatoides are confined to deep water (Anderson, 1994). The intertidal chthamaloids are the oldest group with divergence from the Scalpellomorpha estimated at 93-99 million years ago (Pérez-Losada et al., 2004).

**The barnacle life cycle**

The life cycle of balanomorphs, and other thoracican barnacles, consists of a sessile adult stage preceded by seven larval stages. Figure 1.2 shows the life cycle of a typical balanomorph, *Elminius modestus*. Larval development proceeds from a first-stage non-feeding nauplius, through five planktotrophic nauplius stages, to a non-feeding cypris larva. The planktonic larval phase enables the larvae to disperse, though the distance that individuals may travel is determined by the strength and direction of currents (Crisp, 1974). Benefits of larval dispersal, which favour this essentially r-strategy life history (McArthur and Wilson, 1967), are possibly a wider genetic exchange, the potential to colonise distant geographical areas, the ability to occupy an ephemeral habitat, as well as reduced competition between parent and offspring and between siblings (Crisp, 1974; Pechenik, 1999). In view of the success of barnacles, these advantages would appear to outweigh disadvantages, such as the

| **Phylum: Arthropoda** | | **Subphylum, Crustacea** | |
|---|---|---|---|
| **Superclass: Maxillopoda** | | **Class Thecostraca** | |
| Superorder | Order | Suborder | Family |
| Thoracica | Pedunculata | Iblomorpha | Ibloidea |
| | | Heteralepadomorpha | Heteralepadoidea |
| | | Lepadomorpha | Lepadoidea |
| | | Scalpellomorpha | Scalpelloidea |
| | Sessilia | Balanomorpha | Pachylasmatoidea<br>Chthamaloidea<br>Coronuloidea<br>Tetraclitoidea<br>Balanoidea |
| | | Verrucomorpha | Neoverrucidea<br>Proverrucidea<br>Verrucidea |

Figure 1.1: Taxonomy of the Thoracica (living species) to family level. Source information ERMS (2001) with additional reference to Glenner et al., 1995 and Pérez-Losada et al., 2004.

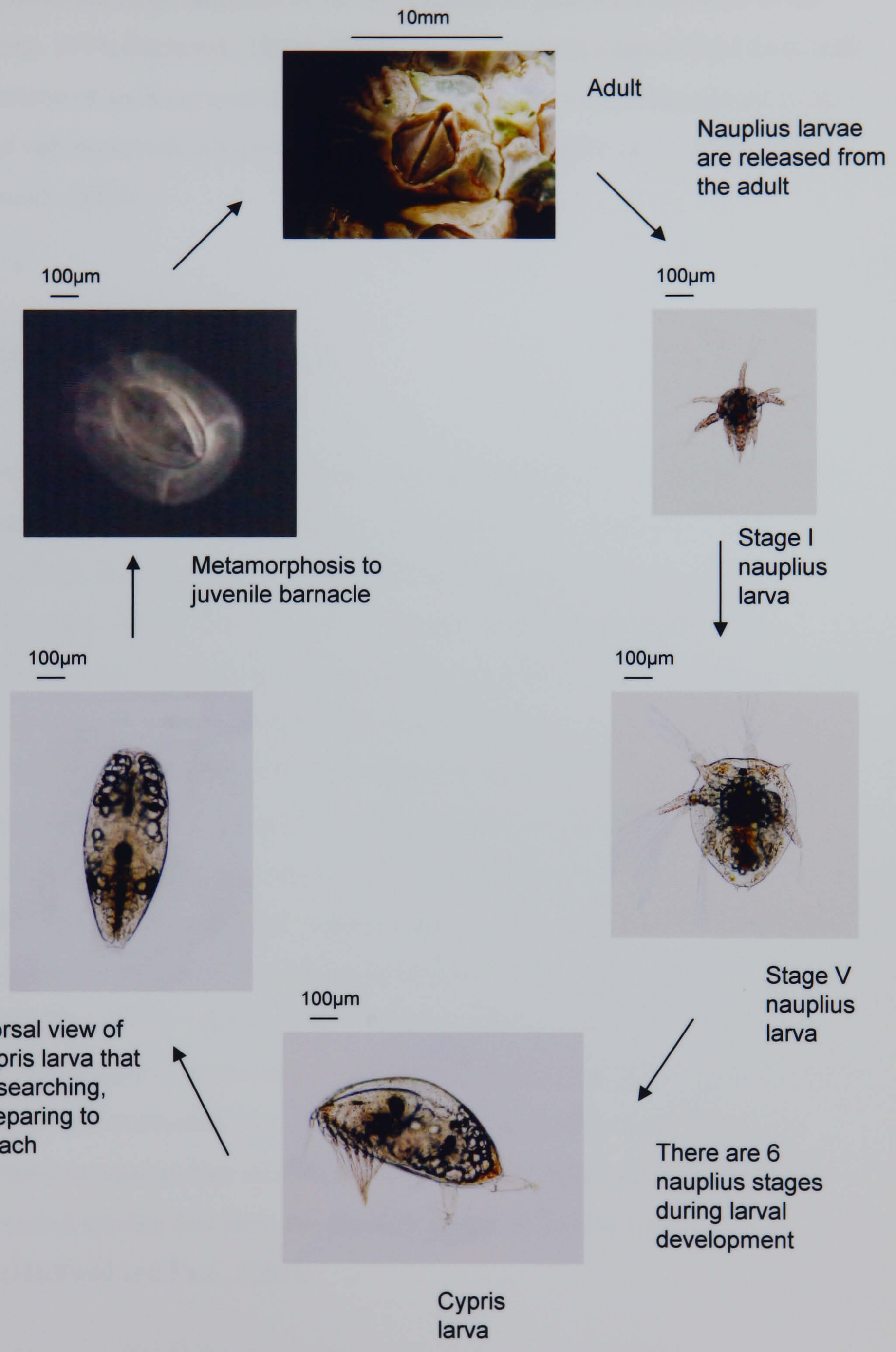



Figure 1.2: The barnacle life cycle, illustrated by the species *Elminius modestus.* Photographs are at different scales – see scale bar above each picture.

risks of predation, high natural mortality due to the planktonic existence and reduced larval fitness if metamorphosis is delayed, as well as the drain on energy reserves of the parent due to the large numbers of larvae required to guarantee survival of the species (Crisp, 1974; Pechenik, 1999). The barnacle cyprid is a specialised form with the sole function of locating a suitable place to attach prior to metamorphosis to the juvenile and subsequent development to the adult form (Walker et al., 1987; Clare and Matsumura, 2000).

## 1.2 Settlement behaviour

Larvae of many benthic invertebrates attach to substrata where they metamorphose into juveniles. Attachment may be temporary, as observed in larvae of mobile organisms such as abalones and sea urchins, or permanent, as is the case for barnacles (Fusetani, 2003). The majority of benthic marine invertebrates have a planktonic larval stage that may last from minutes to months, and significantly may remove larvae away from suitable adult habitats by distances of several metres to hundreds of kilometres (Pawlik, 1992). A variety of cues - physical, biological, and chemical - operating at different spatial scales, are used by many species to enable individuals to find a suitable substratum for the adult phase of their lifecycle. Considerable experimental evidence suggests that chemical cues are very important in substrate selection by larvae, and these are likely to be an important influence once a potentially suitable habitat is reached. Many different species, including polychaetes, echinoderms, bryozoans, ascidians and crustaceans, have been shown to respond to chemical settlement cues. Both waterborne and surface-adsorbed cues have been found in sources that include conspecific individuals, specific prey, and host or associated species that may, for example, provide food or a requisite substratum (Hadfield and Paul, 2001).

Barnacle settlement, being the transition from the free-swimming larva to the sessile adult, is central to the survival and success of the species and is arguably the most important stage of its life cycle (Bourget, 1988). While early ecologists (e.g. Nelson, 1928; Colman, 1933) described settlement as purely a random event in which the

location was brought about solely by the influence of tides and currents, extensive research of sessile marine organisms has identified that larvae exert a degree of control and may delay metamorphosis until suitable habitat conditions are encountered (reviewed by Crisp, 1974). Larvae may distribute themselves throughout the water column (De Wolf, 1973), or may concentrate in surface waters or near the sea floor (Grosberg, 1982; Le Fèvre and Bourget, 1991), which may influence dispersal and shore zonation at settlement (Miron, Boudreau and Bourget, 1995,1999).

**The stages of settlement**

Settlement behaviour occurs once a cypris larva has located a potential habitat and is completed by metamorphosis. The process of settlement has been defined in various ways. Crisp (1984) comprehensively described the whole process. The first stage is attachment and searching, which leads to settlement and is inclusive of various actions:- the cyprid first attaches to the substratum by the attachment disc of the third segment of the antennule; then undertakes wide searching or exploration and 'walks' across the surface by means of its antennules until a suitable location is found; followed by close searching, in which the area is limited and an individual moves more slowly, with many changes of direction; then inspects a confined area and 'steps' to and fro; and finally orientates and settles when a suitable location is decided. The second stage is fixation, in which secretions from the cyprid cement gland irreversibly attach the individual to the substratum. The third stage is metamorphosis of the cyprid to the juvenile form. Lagersson and Høeg (2002) recorded that, during wide searching, *Balanus amphitrite* cyprids walk in straight lines with only minimal changes in direction for periods of 10 seconds to 8 minutes without detachment. During close searching, they observed 'tight-direction changes', whereby the cyprid rotates up to 180° on one attached antennule. While Walley (1969) proposed a two-stage metamorphosis - from nauplius to cyprid, then cyprid to adult - and thereby inclusive of settlement, Pawlik (1992) defined settlement as "the overall process of transition from planktonic larvae to benthic juvenile" and therefore inclusive of metamorphosis. Generally, settlement is understood to be inclusive of metamorphosis, although with significant post-settlement mortality it does not define recruitment, that is those juveniles that survive and go on to form the adult breeding population (Connell, 1985).

# 1.3 Gregarious settlement

### The gregarious response

Gregarious settlement by marine larvae was initially described for the oyster, *Ostrea edulis* (Cole and Knight-Jones, 1949), although earlier observations had suggested that the behaviour existed (e.g. Burton, 1949; Spärck, 1949). Gregarious settlement of barnacles was first described by Knight-Jones and Stevenson (1950) for *E. modestus*. They conducted field experiments in which they exposed glass plates to settling barnacles for 2 to 3 days. Then, by removing spat and cleaning designated areas of each plate, they determined that larvae settled preferentially on areas where conspecifics remained. As they could not rule out the possibility that larvae had responded to a cue from a developing biofilm on the barnacle-settled areas, further experiments were undertaken in the laboratory. Using wild *Semibalanus balanoides, B. crenatus* and *E. modestus* larvae, they showed that cyprids settled readily near to conspecific adults or remnants of their bases on the substratum, though results were not analysed statistically (Knight-Jones, 1953a).

Significantly, Crisp (1990) proposed that barnacle "gregariousness does not simply mean found in a group, but implies the probability that cyprid settlement is increased by the presence of one of its own species". Gregarious settlement is central to the reproductive success of the species. For many species cross fertilisation by copulation is obligatory (Anderson, 1994), such that penis length denotes inter-individual distance. Additionally, other benefits of gregarious settlement have been identified including location of habitats that have already proved suitable for the survival of the species, enhanced competitive ability, reduced predation effects, reduced juvenile mortality and increased filter feeding efficiency (Gotelli, 1990). Alternatively, gregarious settlement may increase intraspecific competition with an individual being eaten, absorbed or removed by conspecifics, or may reduce fitness of an individual due to competition for resources (Pawlik, 1992). However, the evolution of gregarious settlement of barnacles and many other sessile marine species, suggests that overall the advantages of such behaviour outweigh the disadvantages (Pawlik, 1992).

## Chemical settlement cues of barnacle origin

Although the gregarious settlement in barnacles was first identified more than 50 years ago and also that the presence of barnacles at the point of settlement played a significant role in cyprid behaviour, the precise nature of the active substance remains elusive. Investigations during this period have been shaped by the scientific methods and techniques available at the time, such that the understanding of chemical settlement cues continues to be an evolving science.

Knight-Jones (1953a) was the first to identify a settlement-inducing substance for the species *S. balanoides*. He concluded, following a range of experimental tests, that it was a quinone–tanned protein of the epicuticle. The tests illustrated that the substance could withstand exposure to high temperatures; solvents, such as sea water, tap water, chloroform, toluene, concentrated urea and sodium sulphide; and other chemicals, such as hydrochloric acid, nitric acid, and formaldehyde. The substance could be deactivated, however, by boiling in hydrochloric or nitric acid, or sodium hydroxide, as well as by immersion in sodium hypochlorite or mercuric chloride. Crisp and Meadows (1962, 1963) extracted the active factor from several species, including *S. balanoides, E. modestus* and *Chthamalus stellatus*. They named the substance arthropodin, as it shared certain physicochemical characteristics with the insect cuticular protein of the same name (Fraenkel and Ruddall, 1940). Thus arthropodin was believed to be a single substance characteristic of all arthropod cuticles, although later studies have refuted this. A protein mix, including arthropodin, extracted from adult barnacles is known as Settlement Factor (SF) (Rittschof et al., 1984). As activity was present in separate extracts of the shell, inclusive of the hypodermis, and body tissues, Crisp and Meadows (1963) concluded that the substance was present throughout the body tissues of barnacles. Additionally, Knight-Jones (1953a) had previously shown that fragments of body tissue that cyprids would not normally come into contact with, for example cirri and viscera, as well as squashed whole cyprids, induced settlement. Additionally, settled pinhead barnacles yielded active extracts, which were possibly more potent than those of the adult barnacle (Crisp and Meadows, 1962). This result was consistent with a higher cuticle to body ratio and suggested that the protein was essentially cuticular.

Activity was associated with a water-soluble protein, but it was only effective at inducing settlement when adsorbed onto a surface. As little as one or two hundred molecular layers adsorbed onto a clean slate surface were sufficient to induce cyprid settlement behaviour (Crisp and Meadows, 1962). The adsorption of protein may present a molecular configuration similar to the natural conformation of the protein. The nature of the underlying surface has been shown to modulate settlement and this may be as a result of either different levels of adsorption, or different molecular configurations (Crisp and Meadows, 1963). However, cyprids that were immersed in a concentrated solution of SF did react by falling to the bottom and remaining motionless, and moderately dilute extracts caused some cyprids to change from normal photopositive to photonegative behaviour, suggesting some form of perception of the protein in solution (Crisp and Meadows, 1963). Larman et al. (1982) characterised the arthropodin of *S. balanoides* as a polymorphic system of closely related proteins with iso-electric points between pH 4-6, with active subunits of 5-6 kDa and 18 kDa. Larman (1984) considered it similar to actin, a contractile protein that is sticky and binds other proteins. As lectins failed to abolish the inductive effect of arthropodin, Larman et al. (1982) concluded that the carbohydrate moiety was unimportant to activity. However, later findings question the validity of these earlier conclusions (Matsumura et al., 1998b; Clare and Matsumura, 2000).

Arthropodin was initially identified as the only settlement cue, though further research has identified two additional chemical cues, namely the waterborne cue and cyprid temporary adhesive. The waterborne cue, a water-soluble protein, was first identified as a 3-5 kDa peptide (Tegtmeyer and Rittschof, 1989). However, this initial research tested the allospecific reaction of the *S. balanoides* cue on *B. amphitrite*. More recent evidence, examining the conspecific response of *B. amphitrite,* suggests that the waterborne cue may be less than 500 Da in mass (Clare and Matsumura, 2000). Cyprid temporary adhesive, visualised as footprints after cyprids had crossed a surface by means of their antennules (Walker and Yule, 1984), is a further proteinaceous material found to have a secondary function as a settlement cue (Yule and Walker, 1985; Clare et al., 1994). Yule and Walker (1987) showed that in settlement assays with *S. balanoides* cyprids, and using slates that had been 'walked' over by cyprids, settlement was markedly increased by the presence of 'footprints'.

An important outcome of the Fusetani Biofouling Project (Fusetani, 1998) was the development of a nitrocellulose membrane assay, in which protein extracts are attached to the membrane in discrete areas using a modified dot-blotter. Several proteins or protein fractions may be used in a single assay, which has enabled a choice assay to be undertaken in the laboratory. Additionally, it has also been shown to have utility in field studies (Matsumura et al., 2000). The assay was central to the purification of arthropodin from a crude extract of the species *B. amphitrite*. Bioassay-directed chromatography revealed a glycoprotein in excess of 200 kDa molecular mass (Matsumura et al., 1998a). Subsequent analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) determined that the settlement factor comprised three major subunits of 76, 88 and 98 kDa. The subunits, when excised and bioassayed separately, had comparable settlement activity to the intact molecule. Each subunit was found to have lentil lectin-binding sugar chains and, as settlement-inducing activity was suppressed by lentil lectin, the carbohydrate moiety of the protein would seem of importance to *B. amphitrite* (Matsumura et al., 1998b). Matsumura et al. (1998a) introduced the term settlement-inducing protein complex (SIPC) for the purified settlement factor of *B. amphitrite*.

Immunological studies, using a polyclonal antibody raised against the 76 kDa subunit of SIPC, have detected similar protein subunits for 14 barnacle species, indicating a shared epitope (s) (A.Clare pers. comm.; Clare et al., 1997), but with small differences in the molecular mass of reactive bands and intensity of staining in Western blots. Clare et al. (1997) immunostained extracts for five species, *B. amphitrite, S. balanoides, C. stellatus, C. montagui* and *Euraphia depressa*. The results indicated that the reactive band of *S. balanoides* had a higher molecular mass than that of *B. amphitrite* and was in contrast to the findings of Larman et al. (1982). *C. stellatus and C. montagui,* with similar morphology indicative of a close relatedness, had differences in the intensity of staining and slight differences in molecular mass, suggesting some differences in the structure of the subunits (Clare et al., 1997). A mixture of extracts from these two chthamalids produced two distinct reactive bands within a single lane of a Western blot (A.Clare pers. comm.).

Similarly, Kato-Yoshinago et al. (2000) identified SIPC-like protein subunits in seven barnacle species, namely *Mitella mitella, C. challengeri, B. eburneus, B.*

*albicostatus, Megabalanus rosa* and *Tetraclita japonica*. The immunological study also tested a number of non-barnacle species including sponges and molluscs. Although certain non-barnacle extracts were shown to induce settlement, SIPC-like proteins were not detected. For *B. amphitrite*, the polyclonal antibody to the 76 kDa subunit has also detected SIPC-like proteins in extracts of whole larvae, with gradual increases in expression during larval development, reaching a maximum in the cyprid (Matsumura et al., 1998c). Extracts from separated body parts indicated that SIPC was present in the shell with the hypodermis, and in the prosoma (Matsumura et al., 1998c). Additionally, cyprid temporary adhesive, deposited by cyprids as 'footprints' during surface exploration, reacted with the antibody, suggesting an immunological relationship (Matsumura et al., 1998c).

**Chemical perception**

The cypris larva explores a surface using its paired antennules. The antennules also serve as a conduit for the cyprid cement, which attaches the cyprid permanently to the substratum. Further putative functions of the antennules have been proposed, reflecting the impressive array of setae on the various articles. Certain setae on the fourth article are putative chemoreceptive structures. They are flicked across the surface during searching behaviour (Gibson and Nott, 1971; Clare and Nott, 1994; Lagersson et al., 2003). Other setae may be involved in recognising physical characters of the substratum, or may have a dual sensory function. The mechanisms and pathways that enable the cyprid to recognise cues have yet to be established, such that the reception of barnacle chemical cues, that is the adult protein, the waterborne cue and cyprid temporary adhesive, are considered as a single action. However, the possibility clearly exists that these are recognised separately (Clare and Matsumura, 2000).

Nott and Foster (1969), in support of Knight-Jones (1953a), suggested a method of chemo-reception in which proteolytic enzymes released from the antennular disc fragmented the settlement protein, so that the hydrolysate might then be recognised by an olfactory sense. Crisp (1974) proposed the concept of a tactile chemical sense, whereby the cyprid recognises an inductive surface as an increase in adhesion or friction when the attachment disc is in contact with the settlement factor. However, this theory fails to explain how cyprids differentiate between con- and allo-specific

extracts with highly comparable adhesive qualities (Pawlik, 1992), or the fact that they can detect the presence of the settlement factor mixed into gelatin, a substance that does not promote settlement (Crisp, 1984). Crisp (1985) proposed a chemo-sensory mechanism for cue reception in which increased adhesion was a consequence rather than a cause of pheromone recognition.

The axial sense organ, which terminates as an open ended cuticular hair in the centre of the attachment disc and the post axial sense organ at its side, as well as other setae, may be involved in chemical recognition through direct contact with an active surface (Nott and Foster, 1969). Alternatively, while certain setae on the third segment may detect surface roughness by the brushing of the tip over the substratum, and others may be affected by resistance offered to water movement relative to the larvae (Gibson and Nott, 1971), the thin-walled and possible open-ended structures of others could be indicative of chemo-reception, although mechano-reception is not precluded (Gibson and Nott, 1971). Although settlement activity has been observed following physical contact with the active factor, this could be brought about by detection by the setae in the boundary layer above the substratum (Clare and Nott, 1994). The isolation and characterisation of the settlement factors are required to pursue site-specific reception through ligand-binding studies (Clare, 1995).

**Con- and allo- specific settlement**

Knight-Jones (1953a) concluded from laboratory experiments that cyprids only settled readily near to conspecific adults or the remnants of bases on the substratum. Further studies (Knight-Jones, 1955; Daniel, 1955) illustrated that settlement may also be induced in the presence of allospecific barnacles. Knight-Jones and Moyse (1961) observed subjectively that settlement was denser around allospecifics, and concluded that cyprids avoided adults of their own species, thereby reducing intraspecific competition. However, in laboratory experiments they found that *E. modestus* extract stimulated *B. crenatus* settlement to an equal degree, while extracts from Verrucidea and Chthamalidea species had no significant effect on *S. balanoides* settlement. For their experiments, *Mytilus* shells and pieces of slate, were placed in seawater-filled crystallising dishes and settlement on clean surfaces and those with barnacles, or remnants of barnacles, was scored. Uniformity between assays was questionable and additionally, their results were not analysed statistically.

Crisp and Meadows (1962) established that plastic replicas of adult shells and bases did not induce settlement. They then carried out experiments using slate panels in a rotating seawater trough. They found that *S. balanoides* cyprids 'chose' surfaces treated with extracts of conspecific adults in preference to those treated with *E. modestus* extract. Conversely, *E. modestus* cyprids showed a greater response to slates treated with the allospecific extract of *S. balanoides* than that of their own species, though, again, results were not analysed statistically. Larman and Gabbot (1975), using the same rotating trough method, also showed that *S. balanoides* cyprids preferred a conspecific extract to that of *E. modestus*, but conversely that *E. modestus* cyprids did not discriminate between extracts. Again, however, results were not analysed statistically. The results of a field study (Barnett et al., 1979), in which metamorphosed spat in defined quadrats on a rocky shore were counted, indicated that both *S. balanoides* and *E. modestus* cyprids settled in a significantly higher density close to conspecific adults than allospecific individuals. A complementary laboratory study with statistical analysis (Barnett and Crisp, 1979) confirmed the field results. Crisp and Meadows (1962) measured the threshold concentration of crude extracts of *S. balanoides* and *E. modestus* required to induce settlement in each of these species. While it was difficult to compare species directly, due to the unknown quantity of active factor in each extract, they demonstrated that generally conspecific settlement was induced at lower concentrations of extract than allospecific settlement. Crisp and Meadows (1963) reported that *S. balanoides* cyprids exhibited a decline in specificity for conspecific adult extract with age.

More recent research has been centred on *B. amphitrite* using laboratory reared cyprids. This tropical/semi-tropical fouling species has been favoured for research due to relatively easy laboratory culture of adults, the ability of adults to produce a year-round abundance of larvae and a short larval culture period (Clare, 1996). At the same time as the switch to *B. amphitrite*, experimental design has evolved, while the statistical analysis of results has become routine. Crisp (1990) determined that while *B. amphitrite* cyprids responded to the extracts of several species, they settled more readily on conspecific extract than that of four North Carolina (United States) barnacle species. The level of the response varied between species with settlement reduced for more distantly related species. Matsumura et al. (2000) determined that settlement by *B. amphitrite* cyprids was significantly greater to a conspecific adult extract than that of *S. balanoides*. Additionally, settlement by wild *S. balanoides*

cyprids was significantly greater to the conspecific adult extract than that of *B. amphitrite*. In a further study (Kato-Yoshinago et al., 2000), conspecific settlement by *B. amphitrite* cyprids was equal to allospecific extracts of *B. eberneus* and *M. rosa*.

**Extracts from other taxa**

While Crisp and Meadows (1962) established that extracts from a range of marine plants did not induce barnacle settlement, they reported settlement-inducing activity from various arthropods, including *Artemia salina*, *Carcinus maenas* and a non-marine *Blaberus* sp., supporting the relatedness of integumentary proteins within these arthropod taxa. However, they additionally recorded induction of settlement by non-arthropod species, including the molluscs *Nucella lapillus, Mytilus edulis* and *O. edulis*, as well as the fish *Lipophyrys pholis* (previously *Blennius pholis*). Similarly, extracts from the adductor muscles of *M. edulis* and *O. edulis* (Larman and Gabbott, 1975) induced notable barnacle settlement with a greater response by *E. modestus and B. crenatus* cyprids than those of *S. balanoides*. The level of settlement appeared comparable to *S. balanoides'* response to allospecific barnacle extracts. In contrast, Larman (1984) found extracts of the adductor muscle of *Pecten maximus* and the carapace of *C. maenas* promoted settlement by *S. balanoides* cyprids as effectively as conspecific extract.

More recently, a polyclonal antibody to the 76 kDa subunit of *B. amphitrite* SIPC has been used to assay extracts of other species for SIPC-like composition in order to provide a further understanding of these earlier investigations (Kato-Yoshinaga et al., 2000). However SIPC-like proteins were not detected in any of the extracts tested, which included sponges, bryozoans, branchiopods and molluscs. As the Kato-Yoshinaga et al. (2000) study did not replicate the earlier studies (Crisp and Meadows, 1962; Larman, 1984) either by the barnacle species used in settlement assays, or the non-barnacle species used for extracts, and also did not carry out bioassays, it is difficult to compare the findings. The possibility remains that the selected species of the recent study may not induce settlement, despite close taxonomic relationships with species used in the earlier studies. The mechanism by which non-barnacle extracts may induce settlement remains open to question. Recent immunological studies (Kato-Yoshinaga et al., 2000; Matsumura et al., unpublished)

using the 76 kDa polyclonal antibody to *B. amphitrite* have concluded that SIPC-like proteins are specific to barnacles and that settlement is induced by allospecific proteins because they are sufficiently similar, or the SIPC receptor is broadly-tuned (Clare and Matsumura, 2000).

# 1.4 Other factors influencing settlement

Many other factors influence barnacle settlement. While cypris larvae remain essentially passive to stochastic forces (Butman, 1987) brought about by weather-related events and local hydrodynamics, different settlement cues - physical, biological and chemical - affect cyprid behaviour. Behavioural patterns include the 'instinct' to leave the plankton, to first select a generally favourable habitat and then a specific settlement site.

## Environmental influences on settlement

Although chemical cues are important to barnacle settlement (e.g. Thompson et al., 1998), larvae are subjected to various naturally occurring factors, which may influence settlement behaviour. Physical characteristics, such as surface texture (Barnes, 1956; Hills and Thomason, 1998) and surface contour (Crisp and Barnes, 1954), may influence settlement. Holmes et al. (1997) observed that, in field experiments with 15 different rock types, *S. balanoides* cyprids settled preferentially on finely grained (0.002 - 0.006 mm) rock surfaces. However, Caffey (1982) determined that settlement of *Tesseropora rosea* was not affected by different rock types. Generally, cyprids show a preference for concavities and pits, although local environmental factors may be influential. A comparative study of settlement patterns of *S. balanoides* on the Gulf of St Lawrence and the Canadian Atlantic coast (Chabot and Bourget, 1988) revealed that on the Gulf of St Lawrence more individuals settled in crevices, while the reverse was the case for the Atlantic coast. The authors considered that environmental influences may account for the difference, although they suggested that strong selective pressure may have brought about genetic variation that resulted in the preference. Turbulent mixing at a range of spatial scales can either disperse or aggregate larvae (De Wolf, 1973), while at a

reduced spatial scale, flow velocity and direction (Mullineaux and Butman, 1991) are also influential. The effect varies dependent on the species. For example cyprids of *S. balanoides*, an open coast species, are able to continue exploration in stronger shear forces than those of *B. amphritrite*, an estuarine species (Anderson, 1994).

Salinity may also influence settlement. *B. improvisus*, generally found in brackish waters of ports and estuaries (Southward and Crisp, 1963), settled more readily at moderate estuarine salinities than at relatively high or low salinities (Dineen and Hines, 1991). Different microbial or microalgal films on surfaces may stimulate, or more frequently inhibit, settlement (Maki et al., 1988, 1990, 1992; O'Connor and Richardson, 1998; Olivier et al., 2000; Lau et al., 2003), although the age of the film and/or the cyprid (Harder et al., 2001 a and b; Olivier et al., 2000) may be influential. Additionally, the settlement effect of a biofilm may be altered by seawater salinity (Anil and Khandeparkar, 1998). Biofilm effects may be due to changes to surface 'wetability', as well as biological factors and chemical signals from the microorganisms (Walker, 1995). Additionally, Neal et al. (1996) found that *E. modestus* attachment was increased on biofilms formed in high shear rates, which may positively influence settlement. Mucus from other organisms may have an influence. Stimulatory effects have been reported for limpets, nudibranchs and whelks (Raimondi, 1988), and inhibitory effects for cnidarians, limpets and whelks (Johnson and Strathman, 1989). Vibration, related in the natural environment for example to wave action within the intertidal zone, has been identified as a further influence on settlement. A vibration frequency of 27 Hz, equivalent to wave action on a sand beach, and therefore an inappropriate substratum for barnacle species, deters settlement (Branscomb and Rittschof, 1984; Rittschof et al., 1992). In addition to natural cues, many artificial cues to larval settlement are known, which include neurotransmitters, inorganic ions, hormones and pharmacological agents that affect cellular signal transduction systems (Holland et al., 1984; Clare et al, 1995; Kon-ya and Endo, 1995; Yammamoto et al., 1995,1996; Knight et al., 2000).

**The relative importance of cues**

The relative importance of the different cues remains unanswered; a reflection perhaps that settlement of larvae of marine invertebrates is extremely variable in the laboratory and in nature (Holm et al., 2000). Crisp (1984) proposed a hypothetical

sequence of light, current, presence of conspecifics, surface contour, surface hardness and proximity of conspecifics affecting cyprid behaviour, although cyprids may be influenced simultaneously by a range of factors at any given time. In laboratory assays (Thompson et al., 1998), *S. balanoides* cyprids demonstrated a settlement preference for surfaces with a mature microbial film, though corresponding field trials showed that the presence of conspecifics overruled cues from microbiota and the example illustrates the importance of conducting field research. The relationship between cues appears complex, and with possible subtle interaction of effects, it would seem likely to remain elusive.

While gregariousness "may be thought of as a response which prevents settlement on substrata, which are unsuitable only because of the lack of conspecifics" (Hui and Moyse, 1987), the statement fails to take account of the complexity brought about by the response of individuals to both con- and allo-specific cues. Barnes and Crisp (1979) suggested that "gregariousness leads to reductions in local diversity, tending to aid the establishment of monospecific zones on the shore". However, settlement may occur outside the zone of the adult by pioneer individuals and attraction of further cyprids (Crisp, 1974), or by individuals responding to allospecific cues. Post settlement environmental stress and biological factors such as competition, predation and grazing may then influence distribution of adults. De Wolf (1973) concluded that settlement density could not be attributed to the gregarious response, but was more likely explained by the aggregation of cyprids in the water prior to settlement.

**Barnacle physiology**

The energy resources of the non-feeding cyprid limit the period that the cyprid can delay settlement. While difficult to study in the natural environment, in laboratory trials Lucas et al. (1979) calculated that *S. balanoides* cyprids remained competent to settle for a period of 3 to 4 weeks. Additionally, settlement increases with age (Rittschof et al., 1984; C.Hellio pers. comm.), though conversely, Satuito et al. (1996) reported an increase in settlement from age 0 to 3 days for *B. amphitrite* followed by a significant decline with additional ageing. In the natural environment, the postponement of metamorphosis in the absence of a suitable habitat may allow an individual to disperse to a location more likely to support its survival and successful

reproduction (Crisp, 1974). However, it is difficult to determine whether an extended laboratory competence is a true indication that postponed metamorphosis occurs in the field. At present little is understood of the occurrence of delayed metamorphosis and the environmental conditions underlying the behaviour (Pechenik, 1990; Jarrett, 1997). Laboratory experiments have shown that postponing larval settlement depressed post metamorphic growth rate (Pechenik et al., 1993). Such deferment in the field may result in larvae that are less able to compete for space. Food resources during larval development may be a further influence. For example, the microalgae used to feed developing larvae have been shown to influence settlement in laboratory conditions (Holm, 1990; Qui and Qian, 1998). A study of the settlement response of *B. amphitrite* cyprids that had been reared on various algal diets of different lipid content (Harder et al., 2001a and b) considered that the combined effect of age and lipid reserves influenced the rate of settlement. In the natural environment, food resources are likely to be of greater variety, with variation in abundance also. This may affect larval development and energy reserves, which may in turn have an impact of the settlement behaviour of individuals.

**Associative settlement**

Many species of barnacle exist in close association with other marine organisms and the term associative settlement was first used by Crisp (1974) to describe the enhanced or specific settlement of one species on another. While species of the Ascothoracica and Rhizocephela are parasitic, thoracican barnacles form relationships that are commensal or symbiotic. Focusing on Balanomorph species, epizoic coronuloids are confined almost entirely to marine reptiles and marine mammals, while the hosts of epizoic balanoids are mainly sessile organisms such as sponges, gorgonians and corals (Anderson, 1994). Figure 1.3 identifies the major hosts of the genera of these two groups.

Settlement patterns on the host organism may vary according to the species. An Australian study of barnacles found on turtle species (Monroe, 1979: in Anderson, 1994) found different forms of attachment (superficial, embedded or burrowing) by different species, as well as many species having a preferred location on the body of the host. For example, *Xenobalanus globicipitis* is restricted to the fins and tail flukes of its cetacean hosts (Spivey, 1980; Rajagura and Shantha, 1992). The

| Barnacle species | Host |
|---|---|
| **Coronuloidea** | |
| *Chelonibia* | Turtles, crocodiles, sirenians, decapod crustaceans, molluscs |
| *Platylepas* | Turtles, seasnakes, sirenians |
| *Stephanlepas* | Turtles |
| *Stomatolepas* | Turtles |
| *Cylindrolepas* | Turtles |
| *Coronula* | Large whales |
| *Cetopirus* | Large whales |
| *Cryptolepas* | Grey whales |
| *Tubicinella* | Turtles, Southern Right whales |
| *Xenobalanus* | Dolphins, porpoises, blackfish |
| **Balanoidea** | |
| *Armatobalanus* | Scleractinian corals |
| *Membranobalanus* | Sponges |
| *Acasta* | Sponges |
| *Conopea* | Gorgonians |
| *Balanus spongicola* | Sponges |

Figure 1.3: Coronuloidea and Balanoidea barnacles and their major hosts (Anderson, 1994 ).

selectivity of host occupancy varies among species and genera, for example *Coronula diadema* lives on most large southern whales, while *Coronula reginae* is restricted to humpback whales only (Anderson, 1994). The degree of selectivity suggests that evolutionary adaptation has occurred and that settlement cues may be involved (Lewis, 1978), as the more peculiar the habitat and the less frequently it is likely to be encountered, the more insistent would be the need for specific recognition (Crisp, 1965). A molecular study of 12S mitochondrial (mt) DNA of *Savignium milleporum* (Mokady and Brickner, 2001), a coral-inhabiting barnacle species with three related hosts, showed that although mt DNA was almost identical within populations of the same host, the divergence between populations was on average 9%, although all individuals appeared morphologically similar. The results are suggestive of sequential evolution of host and symbiont. Certain rhizocephalan species are able to locate their host by distance-chemoreception of waterborne cues (Pasternak et al., 2005).

The presence of SIPC-like proteins in host-symbiont relationships remains to be investigated. As the larvae must initially locate the host, and then locate individuals of its own species (gregarious settlement), it remains possible that SIPC-like proteins act at least in part of this process.

## 1.5 The molecular context

### The opportunities for molecular methods

The use of molecular techniques has rapidly become central to many avenues of biological research. The study of genes has proved to be a powerful approach to further the understanding of biological systems (Griffiths et al., 1999), with perhaps the human genome project viewed as the pinnacle of this increasingly-essential form of analysis. Molecular techniques have numerous applications (Avise, 1993). For example, DNA sequence divergence can indicate different genotypes with the possibility of considering geographic population structure, perhaps indicative of variable environmental parameters, or gene flow within a population. The relatedness of species can be assessed including, at a macroevolutionary level, the

phylogenetic relationships between organisms. Sequencing of appropriate genes allows protein sequences that they encode to be determined with the possibility of further investigating sites and timing of expression.

Examples of molecular studies on barnacle species include the analysis of genes in *M. rosa* that encode cement adhesive peptides (Kamino et al., 1996); molecular cloning of the putative serotonin receptor gene in *B. amphitrite* (Kawahara et al., 2000); the isolation of cyprid-specific genes in *B. amphitrite* that encode peptides that may be involved in attachment and metamorphosis (Okazaki and Shizuri, 2000); and nucleotide sequencing of mt DNA, combined with morphological evidence, to determine the phylogeny of three species of the genus *Pollicipes* (Van Syoc, 1995). Additionally, Pérez-Losada et al. (2004) investigated thoracican barnacle evolution through the comparison of mt DNA sequences from 43 taxa, combined with morphological evidence.

Molecular methods, which enable the characterisation of the settlement protein gene, and thereby its amino acid structure, may be particularly useful in determining how species distinguish between SIPCs. Although the amino acid sequence may be determined for individual species directly from isolated protein, each species studied would require an equal resource investment that could easily prove excessive. Isolation of the gene responsible for SIPC, although initially time consuming for a single species, offers an effective approach to multiple species studies with the possibility of a diminishing input of resources for subsequent species. Additionally, proteins may also be produced in relatively large quantities by recombinant techniques that may then facilitate more in-depth research.

**The settlement-inducing protein complex of *Balanus amphitrite***

Following the isolation of *B. amphitrite* SIPC (Matsumura et al., 1998a), short amino acid sequences were obtained for each of the SIPC subunits and, initially, the 76 kDa subunit of the SIPC for *B. amphitrite* was sequenced through polymerase chain reaction (PCR) experiments using primers designed from the known peptide fragments (A.Clare pers. comm.). The subunit sequenced indicated a composite molecular mass of around 52 kDa. Additionally, the subunit has associated sugars that account for the 10-15% of its total mass (A.Clare pers. comm.). Most recently,

cloning of the SIPC gene in entirety has been achieved and the primary protein structure of *B. amphitrite* SIPC has been determined (A.Clare pers.comm.).

The structure shows similarity to the alpha (2) macroglobulin (A2M) family of proteins (A.Clare pers.comm.). A primary function of A2Ms is the clearance of active proteases involved in pathogenic attack. As such, A2Ms function as part of the innate immune systems of vertebrates, as well as several invertebrate phyla including arthropods (Armstrong et al., 1996). They arose early in the evolution of multicellular organisms and have been preserved through evolutionary divergence of the different animal phyla (Armstrong et al., 1996). A2Ms from the horseshoe crab, crayfish lobster and octopus share many of the unique structural and functional characteristics of mammalian counterparts including significant sequence identity (Hall et al., 1989; Sottrup-Jenson et al., 1990); a reactive internal thioester bond (Armstrong and Quigley, 1987); reactivity with proteases of differing enzymatic mechanisms (Quigley and Armstrong, 1985); the unique protease trapping mechanism (Armstrong and Quigley, 1991); and the ability to participate in the clearance of proteases introduced into the plasma (Melchior et al., 1995). In addition A2M binds a variety of other ligands including several peptide growth factors, and modulates the activity of a lectin-dependent cytolytic pathway in arthropods (Armstrong and Quigley, 1999).

The full sequencing of the *B. amphitrite* SIPC encodes a protein of 1548 amino acids, including a putative 17-residue N-terminal segment indicative of a secreted protein. Database comparisons (Genbank) of the predicted SIPC amino acid sequence revealed that the highest homology was shared with A2Ms of other arthropods, i.e. the tick, *Ornothodoros moubata* (31%) and the horseshoe crab, *Limulus* sp. (29%). A phylogenetic analysis showed that SIPC belongs to a subgroup of A2Ms, distinct from the complement and thioester protein families. However, *B. amphitrite* SIPC does not possess the thioester bond, unique to all known A2Ms, or a 'bait' region, both of which are essential to its function in the immune response. Furthermore sequencing of a fragment of the gene encoding A2M from 3 barnacle species has indicated that they differ from SIPC (C.Dreanno pers.comm.). Thus, it would seem likely that SIPC has diverged from A2M within the organism in the early stages of evolution. Attempts to isolate SIPC from

additional barnacle species, using primers designed from the *B. amphitrite* gene sequence, have been unsuccessful (R.Kirby pers.comm.), suggesting that the gene may not be highly conserved between species.

**Marine chemical ecology**

More than seven thousand natural products have been isolated from a vast range of marine organisms (Harper et al., 2001), an indication of the long-standing scientific interest in chemical synthesis by marine organisms. Around 75% of these compounds are from invertebrates, although the Arthropoda including Cirripedes account for less than 1% of the total. While non-registration of arthropod natural products is a possibility, the low percentage may reflect the proportion of research on this group, rather than an absence of chemical synthesis in the organisms. Natural products already identified include terpines, acetogenins, alkaloids and polyphenolics (Hay, 1996). The function and complete characterisation of many of the compounds have yet to be fully determined. Additionally the source of many products remains unanswered with the possibility of synthesis by intrinsically associated symbionts or dietary sources in many cases. Many are structurally complex compounds, and may be present at high concentrations, which suggests they have a specific function (Pawlik, 2000). There is increasing evidence, however, that many play fundamental roles in the ecology of the marine environment. Reputed ecological roles for marine natural products include anti-predation, mediation of spatial competition, prevention of fouling, facilitation of reproduction and protection from ultra-violet radiation (Harper et al., 2001). Seasonal variation (Paul and Puglisi, 2004) and also intraspecific variation at the individual and population level (Hay, 1996) have been identified for some products, though reasons for variation are not adequately known. Biomedical applications include the development of antibiotics, pain suppressors, anti-inflammatory agents, molecular probes, skin care, sun screens and anti-cancer agents (Hay, 1996). However, while biomedical applications have been actively pursued, the potential of natural antifouling products has yet to be fully exploited. Some marine natural products have been tested for potential industrial antifouling, including halogenated furanones, pukalides and renillafoulins (Fusetani, 2003). The characterisation of SIPC is a first step towards the potential development of a natural antifoulant.

## 1.6 Conclusion

### 1.6.1 Key findings

Gregarious settlement by barnacles was first established through field experiments by Knight-Jones and Stevenson (1950) and confirmed by laboratory experiments using wild *E. modestus* cyprids. Settlement assays have illustrated that species respond to both con- and allo-specific extracts and that they react differently to extracts of their own and other species. However, comparisons of previous investigations, particularly early experiments using temperate species, are contradictory and statistical analyses have been limited. More recent research has favoured the use of laboratory-reared *B. amphitrite* cyprids, such that our current understanding of barnacle settlement behaviour is dominated by *B. amphitrite* experiments. Current understanding may be enhanced by the reinvestigation of the behaviour of temperate species using more recently developed methods combined with statistical analyses. Additionally, the opportunity remains to investigate con- and allo-specific settlement further using a wider range of allospecific cues in a single experimental design. While it would be ideal to undertake investigations using purified SIPC, not only is the present method of isolation laborious, it is reliant on the results of conspecific bioassays to isolate the relevant protein (2.4.2). To date, however, only a limited number of species have been successfully cultured to the cyprid stage. Thus, in order to broaden investigations, the use of similarly prepared partially purified extracts, combined with modern assay methods and statistical analyses, is preferred. In the longer term, when advances in research may permit more routine isolation of SIPC, the opportunity remains to more accurately define the relationships through the use of isolated settlement-inducing proteins for both con- and allo- specific settlement. While earlier experiments have been conducted in both the laboratory and the natural environment, there is the opportunity to develop field experimental methods that are comparable to laboratory techniques, so that laboratory and field results may be compared more effectively. Additionally, the presence of SIPC-like proteins in host-symbiont interactions of associative settlement has yet to be investigated.

The full sequence of the SIPC gene of *B. amphitrite* has been established, though it has not been possible to use the sequence information to isolate SIPC from further species. Therefore, the next step is to isolate SIPC and determine the full genetic sequence from at least one further barnacle species. Then, the two SIPC genes may be aligned and conserved areas of the gene identified. This would improve primer design and, in turn, may lead to a more routine isolation of SIPC from barnacles. SIPC function in con- and allo-specific interactions may then be investigated at the molecular level. Sequencing of *B. amphitrite* has shown that it belongs to a subgroup of A2M proteins, appropriate functional studies are required to determine if *B. amphitrite* SIPC is separate from the immune function.

## 1.6.2 Research opportunities

Research opportunities are identified as follows:-

1. The further investigation of settlement behaviour of temperate species using laboratory-cultured larvae of known age, combined with more recent assay methods and a statistical analysis.
2. The further investigation of con- and allo-specific settlement using adult cues from a wider range of species in a single experiment.
3. The development of a field assay method to improve comparisons of results of laboratory and field experiments.
4. The isolation of SIPC from a second barnacle species, and the sequencing of the appropriate gene, in order to identify conserved sequences and to improve understanding of con- and allo-specific settlement behaviour.
5. More detailed investigations of SIPC in the context of barnacle physiology, for example, to answer questions such as:-
   - Do different species of barnacle express the same amount of SIPC and where within the organism is it expressed?
   - Is there seasonality to SIPC production for some species i.e. comparing a species with a single annual reproductive event to one with continual reproduction?

- Do SIPC-like proteins function in host-symbiont interactions, either between adult and cyprid or host and cyprid, and what does this indicate about the evolution of such relationships?
- Is SIPC separate from the immune function?

## 1.6.3 Research aims and objectives

Items 1 to 4 were selected for further investigation and the following aims and objectives were defined:-

**Aim 1**

To investigate the utility of *E. modestus* and *S. balanoides* as a model temperate species for laboratory assays.

**Objectives**

1.1 To explore workable practices for the routine laboratory culture of larvae.

1.2 To explore the practicality of assay methods, including the 24-well plate and nitrocellulose membrane assay.

1.3 To investigate parameters for assays, including cyprid age, temperature, assay duration and control treatments.

**Aim 2**

To progress the understanding of SIPC at the molecular level.

**Objectives**

2.1 To isolate the SIPC of *E. modestus* in order to obtain a partial amino acid sequence for primer design.

2.2 To determine using molecular biological techniques the primary sequence of a subunit of the SIPC of *E. modestus*.

**Aim 3**

To investigate the settlement response of cypris larvae of selected species to con- and allo-specific SFs in controlled laboratory and field conditions.

**Objectives**

3.1 To investigate, by laboratory assays, the settlement behaviour of *E. modestus* in response to con- and allo-specific SFs.

3.2 To investigate, by field experiments, the settlement behaviour of *S. balanoides* in response to con- and allo-specific SFs.

3.3 To undertake a field experiment trial as in Objective 3.2 for *E. modestus*.

# Chapter 2

# Materials and methods

# 2.1 Introduction

A variety of materials and methods were used during the research project. This chapter is a summary of the principal techniques used, while specific parameters, experimental design and some minor techniques are described in the appropriate chapter. This chapter comprises four sections:-

- Laboratory settlement assay techniques (2.2).
- Field settlement assay techniques (2.3).
- Protein-related protocols (2.4).
- Molecular biology-related protocols (2.5).

# 2.2 Laboratory settlement assay techniques

Laboratory settlement experiments used the 24-well plate and the nitrocellulose membrane assays.

## 2.2.1 24-well plate assays

The 24-well plate assay was a modification of the Petri dish assay described by Rittschof et al. (1992). Multiwell plates, with a maximum well capacity of 2.5 ml (24-well sterile non-treated polystyrene, Iwaki, Japan), were used. Different treatments in 0.45 $\mu$m-filtered seawater were introduced into a designated number of wells with generally six or eight replicates of each treatment per experiment. The treatment was introduced into the well by one of two methods: 1) The concentrated solution was placed in the bottom of each well and the plate swirled for 2 minutes, so that the treatment made contact with the inner surface. Filtered seawater was then added to a total volume of 2 ml; 2) The concentrated solution was added to a measured volume

(sufficient for all replicates) of filtered seawater in a disposable centrifuge tube, which was then vortexed to ensure uniform mixing. Aliquots of 2 ml of the prepared seawater were placed in wells as appropriate (Figure 2.1). After initial assays, the second method became standard practice. Ten cyprids were transferred to each well, the lid was replaced and the plate was then wrapped in aluminium foil to exclude light. The assay was incubated at the experiment temperature and cyprid behaviour was scored at 24±2 hours, and for assays of longer duration, at additional 24-hour intervals. The proportion of cyprids that were settled, dead, floating and swimming was recorded.

## 2.2.2 Nitrocellulose membrane assays

The nitrocellulose membrane assay was a modification of that described by Matsumura et al. (1998a), and it was used specifically to test cyprid behaviour in the presence of adult SF (2.4.2.1). For each assay, nitrocellulose membrane measuring 135 mm x 95 mm (0.45 μm plain white membrane, MFS Advantec, USA) was soaked in 50 mM Tris-HCl, pH 7.5 for 10 minutes, and then sandwiched within a modified dot-blotting apparatus (Atto Immunodot AE-6190, Atto Co. Ltd., Japan) with 10 mm diameter wells (Figure 2.2). SF was diluted in 50 mM Tris-HCl pH 7.5 to the required protein concentration, with 1 ml applied to each of a selection of wells. For the remaining wells, 1 ml of 50 mM Tris-HCl, pH 7.5, or for certain assays bovine serum albumin (BSA, Sigma UK) at the same concentration as the SF, was applied as the control treatment. The apparatus was attached to a vacuum manifold and the membrane was aspirated gently for 10 minutes. This action stretched the membrane slightly in the well areas and resulted in concave circular regions ('spots') on the membrane, a selection of which had been treated with adult protein extract. The membrane was partially dried and fixed to the bottom of a new polypropylene container using double-sided carbon tape (Agar Scientific, UK) around the full perimeter of the membrane (Figure 2.3). Two variations of the modified apparatus were used with either 12 wells in two 3 x 2 arrays, or with 24 wells in a 6 x 4 array. For the former, after application of the treatment the membrane was cut in half resulting in 2 membranes each with 6 'spots'. The treated wells in these

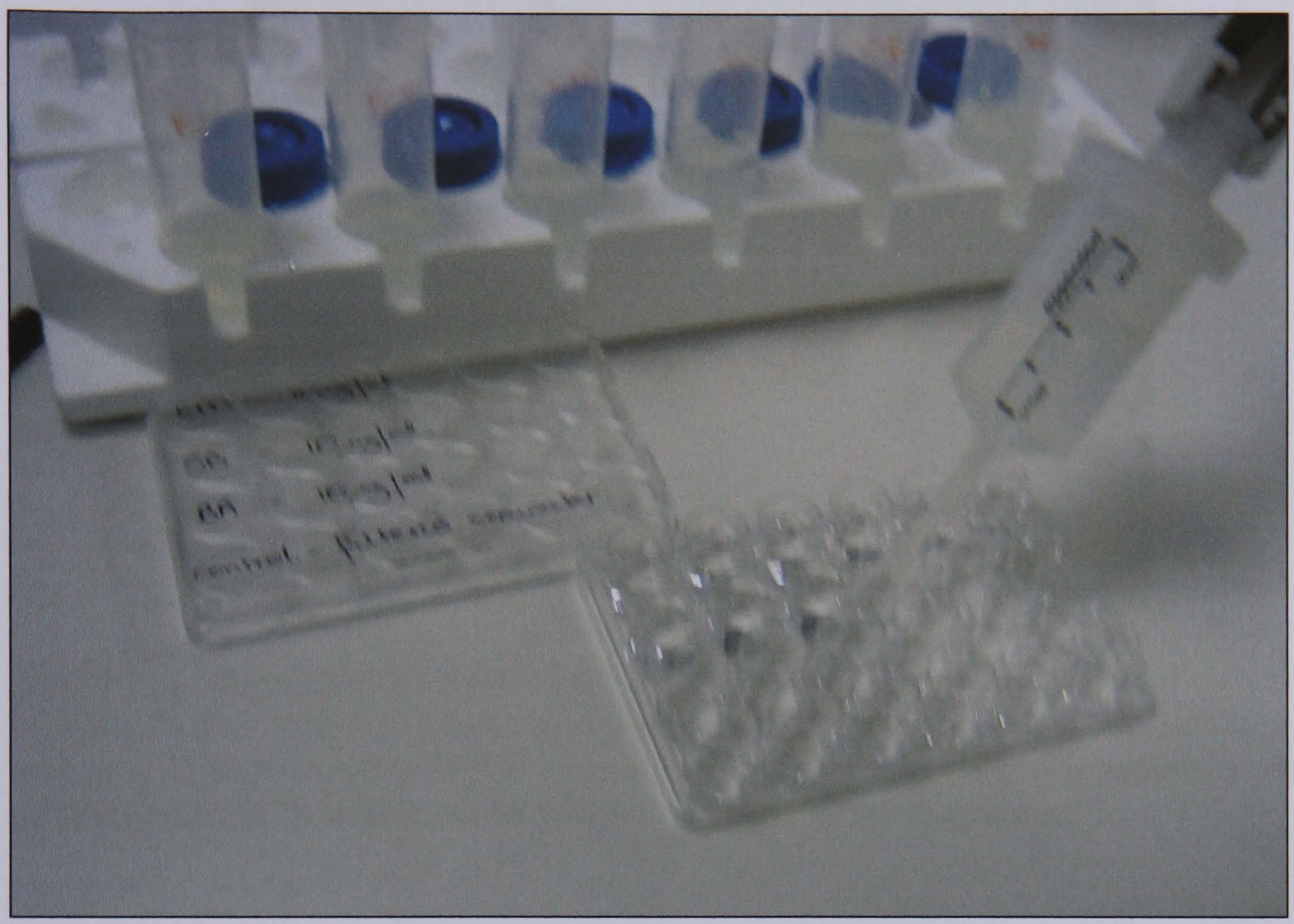


Figure 2.1: Preparation of a 24-well plate assay. The treatment has been suspended in seawater with different concentrations in each of the disposable centrifuge tubes. A 2 ml aliquot is pipetted into each well.

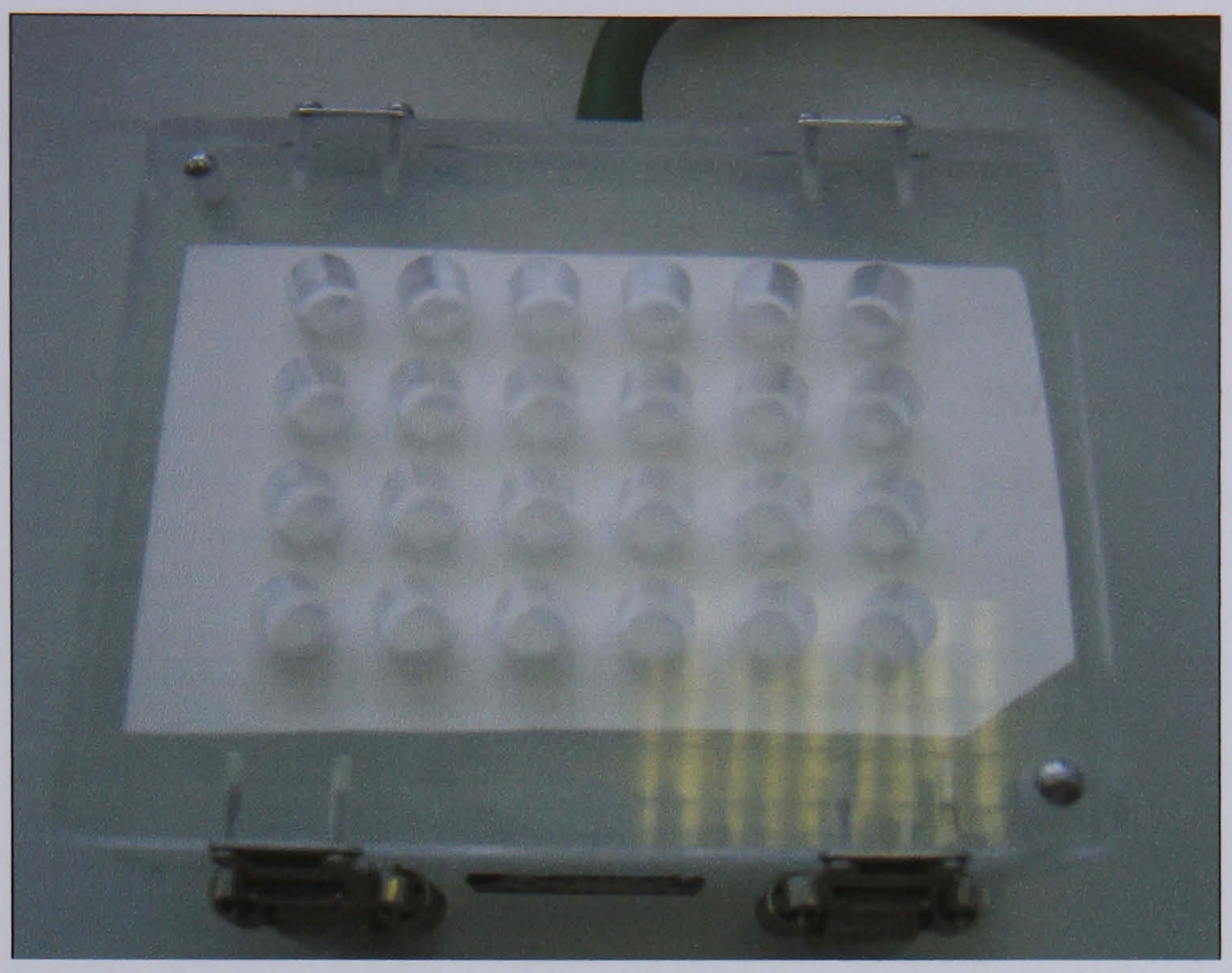


Figure 2.2: 24-well modified dot blotter with nitrocellulose membrane sandwiched inside. The next step is to pipette a 1 ml aliquot of different protein treatments into each well. The apparatus is attached to a vacuum manifold ready for gentle aspiration of the sample through the membrane to attach the protein to the membrane.

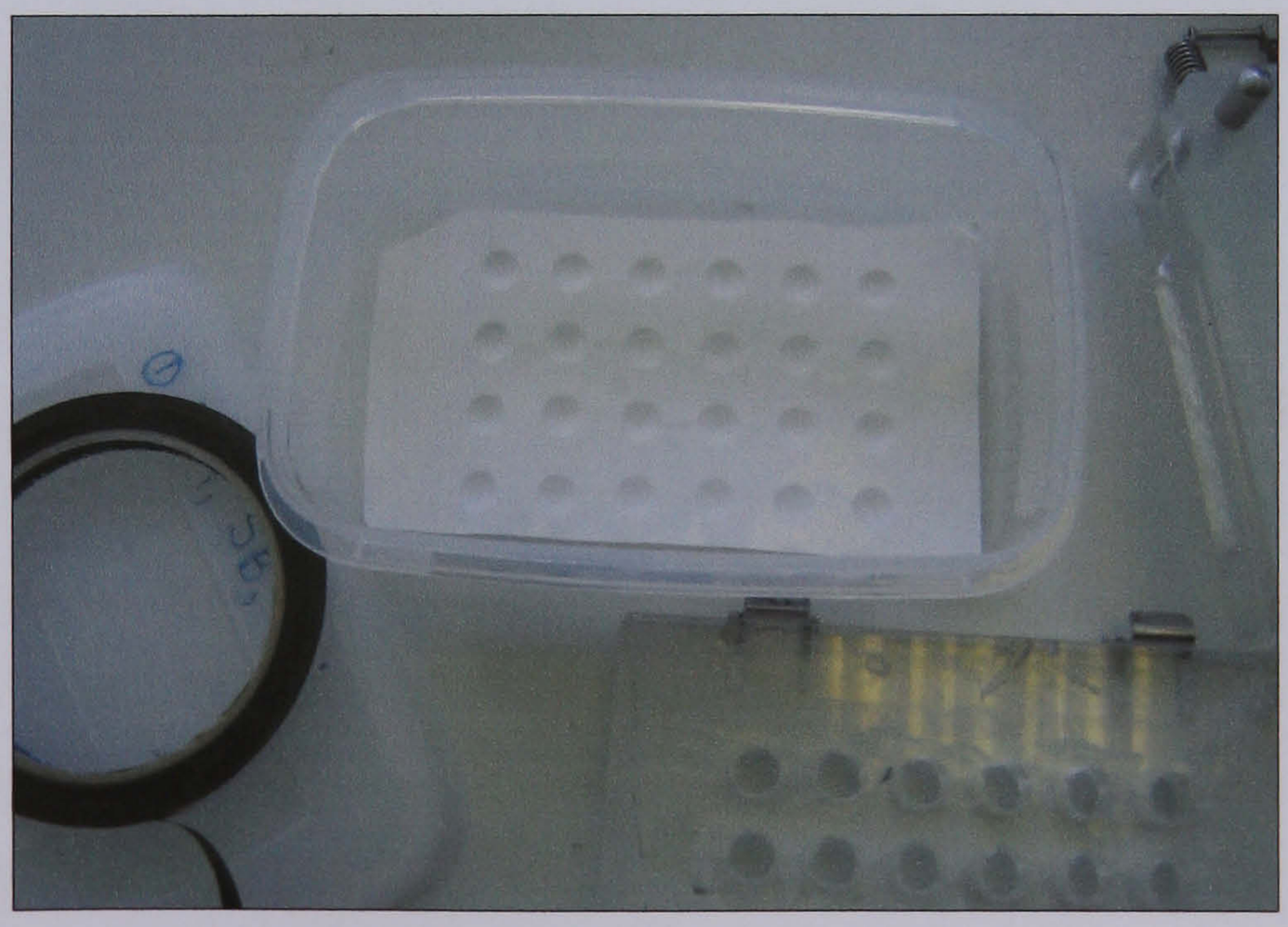


Figure 2.3: The prepared nitrocellulose attached to the bottom of the assay container using double-sided carbon tape.

assays were selected systematically with only one treatment and a control used on a membrane. The assay container measured 100 mm x 55 mm x 65 mm with 340 ml capacity (As One Co. Ltd., Japan), which was partially filled with 200 ml of 0.45 $\mu$m filtered seawater. The membrane prepared using the apparatus with a 6 x 4 array was used as a single 24-spot assay. For all behaviour experiments, the treated wells were selected randomly with between one and five treatments, plus a control, used on a membrane. This assay was also used to isolate *E. modestus* SIPC (2.4.2.2). The assay used a 100 mm x 150 mm x 50 mm (1 litre capacity) container (Whitefurze Ltd, UK), which was partially filled with 400 ml of 0.45 µm filtered seawater. Cyprids were added and the container's lid was replaced. The container was then wrapped in aluminium foil to exclude light and the assay was carried out at the appropriate temperature. Cyprid settlement was scored at 24±2 hours, and for certain assays, at further 24-hour intervals. Settlement was recorded for each concave 'spot' area, and also the 'spot' plus the surrounding area of membrane, by the subjective division of the membrane into 6 or 24 squares.

## 2.2.3 Statistical methods for laboratory assays

### 2.2.3.1 24-well plate assays

For statistical analyses of 24-well plate assays, settlement counts were converted to percentage values after adjusting for dead cyprids. A Kolmogorov-Smirnov test was carried out on the percentage data and arcsine transformed data, to test for a normal distribution, while a Levene's test was carried out similarly to test for homogeneity of variance. Most frequently, both the raw and transformed settlement data were not normally distributed and displayed heteroscedasticity. These results lead to the selection of non-parametric tests for data analysis. Mann-Whitney U tests were used where the test variable had two values, and Kruskal-Wallis tests were used where the test variable had more than 2 values. Where a significant result was obtained from a Kruskal-Wallis test, and it was appropriate to understand which treatments produced significant differences, Dunn's multiple comparisons procedure was undertaken.

### 2.2.3.2 Nitrocellulose membrane assays

For the statistical analysis for nitrocellulose membrane assays, a Kolmogorov-Smirnov test was carried out on settlement counts and data transformations, including log+1, square root, cube root and fourth root (Quinn and Keogh, 2002), to test for a normal distribution. A Levene's test was carried out similarly to test for homogeneity of variance. Most frequently both raw and transformed data were not normally distributed and displayed heteroscedasticity. Non-parametric methods were selected and statistical analysis was carried out as described for 24-well plate assays (2.2.3.1)

## 2.3 Field settlement assay techniques

### 2.3.1 24-well slate assay

The nitrocellulose assay method was adapted for use in field experiments. The contour of a prepared nitrocellulose membrane was replicated on slate. Slate panels, measuring 160 mm x 80 mm x 8 mm, were cut from roofing slates (Cleveland Roofing, UK). A 6 x 4 array of 10 mm diameter concave wells was drilled into the surface of each slate using a bull-nosed slotting drill. Additionally a hole was drilled through either end of the slate for attachment to a substratum. The slates were prepared for the experiment in the laboratory before placing them on the shore at low tide. Wells were treated randomly with SF (2.4.2.1), or were left blank. The SF was diluted to 1 mg $ml^{-1}$ and applied to each well using an artist's paintbrush, with separate identical brushes used for extracts of different species. The wells were allowed to dry in a horizontal position before the slates were moved. In trial experiments, the slates were used individually and were screwed directly onto rocks on the shore. In full-scale experiments, nine slates, in a 3 x 3 array, were attached to a backing panel of Perspex or plywood, using nylon nuts and bolts (RS Components, UK). The panel was then attached to the shore using ropes and cable ties. Settlement was recorded at intervals of two tidal cycles for 24, 48 or 72

hours. For 24-hour experiments and the final observations of the experiments of a longer duration, settlement was recorded in the laboratory by viewing under a binocular microscope. For the other observations of 48- and 72-hour experiments, settlement was recorded in situ using a hand lens. The slate panels were cleaned thoroughly at the end of each experiment prior to re-use. Settled cyprids were removed from the slates using paper towel. The slates were then soaked in a 5% sodium hypochlorite solution (Simply Bleach, Kwik Save, UK) for a minimum of 12 hours, scrubbed clean under running water using a nylon brush, then heated to 150°C for 120 minutes and allowed to cool before re-use.

### 2.3.2 Field testing of nitrocellulose membranes

The durability of the nitrocellulose membrane assay in field experiments was tested. The 24-spot nitrocellulose membranes were prepared as described for laboratory assays (2.2.2). The membrane was allowed to dry and was then placed on top of a clean 24-well slate. The membrane was trimmed to the exact size of the slate using a razor blade, so that the concave wells of the membrane rested in those of the slate. Elastic bands were placed around both ends of the slate, and it was attached to a Perspex or plywood back panel, which in turn was attached to the shore using ropes and cable ties (2.3.1). Settlement was scored as described in Section 2.3.1.

### 2.3.3. Statistical methods for field assays

The statistical analysis of the trial assays, conducted in 2002, is described in Chapter 7. These results have been used to inform the experimental design of full-scale experiments. For the statistical analysis of slate-panel field assays, a Kolmogorov-Smirnov test was carried out on settlement counts and data transformations, including log+1, square root, cube root and fourth root (Quinn and Keogh, 2002), to test for a normal distribution. A Levene's test was carried out similarly to test for homogeneity of variance. Most frequently both raw and transformed data were not normally distributed

and diplayed heteroscedasticity. Non-parametric methods were selected and statistical analysis was carried out as described for 24-well plate assays (2.2.3.1). Statistical analysis for the trial nitrocellulose assays was carried out as described for the laboratory nitrocellulose assays (2.2.3.2).

# 2.4 Protein-related protocols

## 2.4.1. General methods

### 2.4.1.1 Total protein assay

The total protein assay was used to measure the protein content of samples prepared from barnacle tissue. The protein content was estimated by calibrating a colour change of dye (Total Protein Reagent, Bio-Rad, UK) following the manufacturer's protocol. Briefly, the dye was added to the sample and compared to a serial dilution of BSA (Sigma, UK) that had the same dye concentration. The comparison was undertaken spectrophotometrically at a wavelength of 595 nm. Results were only accepted when the accuracy of the calibration curve, as indicated by $r^2$, was greater than 0.998, and the estimated protein, when diluted, was within the protein standards range.

## 2.4.1.2 Gel electrophoresis (SDS-PAGE) and associated methods

### 2.4.1.2.1 Standard gels

SDS-PAGE was normally performed using a 10% acrylamide (30% Acrylamide/BIS 29:1, Bio-Rad, UK) slab gel with a 4.5% stacking gel (Laemmli, 1970). Occasionally, concentrations of acrylamide between 7.5 and 12% were used. Electrophoresis was generally carried out using a minigel system (Atto Co. Ltd, Japan) following the manufacturer's instructions. The total size of the gel was 90 mm x 80 mm x 1 mm, and a 12-compartment comb was placed in the stacking gel to create wells with a maximum 40 $\mu$l capacity. A large gel system (Atto Co. Ltd., Japan), 135 mm x 130 mm x 2 mm, with an 8-compartment comb used to form wells of a maximum 200 $\mu$l capacity in the stacking gel, was used occasionally. The electrophoresis sample was prepared by boiling the protein sample for 5 minutes in Laemmli sample buffer (0.0625 Tris-HCl pH 6.8; 10% glycerol; 2% SDS; 5% 2-mercaptoethanol; 0.1% Bromophenol blue). The ratio of protein sample to buffer was such that all prepared samples had a final 1x concentration of the Laemmli buffer. A 2x Laemmli buffer was used, except when the protein concentration of the sample was low, when 5x buffer was used. Molecular weight standards (Sigma high range markers or Bio-Rad pre-stained broad range markers) were used in one or two lanes. Unless used for Western blotting, the gel was stained with Coomassie Brilliant Blue (0.025% Coomassie Brilliant Blue; 50% methanol; 10% glacial acetic acid) and the protein bands were visualised by placing the gel in a de-staining solution (25% methanol; 10% glacial acetic acid).

### 2.4.1.2.2 Gradient Gels

Gradient gels, with a 5-15% gradient and 4.5% stacking gel (total size 135 mm x 130 mm x 2 mm) were run using a large gel system (Atto Co. Ltd., Japan) following the manufacturer's instructions. The gradient gels were prepared using a gradient former and the gel mixture was slowly trickled into position between the plates using a peristaltic pump. Two types of gradient gel were tested: a linear gradient gel, to give

improved definition of bands, and an exponential gradient, to increase the distance between bands. For the linear gradient, equal volumes of the higher (20%) and lower (5%) concentrations of gel were prepared. The higher gel concentration was placed in the direct outflow container of the gradient former. For the exponential gradient, 1 volume higher to 3 volumes lower concentration of gel were prepared. For both gels, the concentration was highest at the base of the gel and lowest at the top.

#### 2.4.1.2.3. Western blotting and immunostaining

The SDS-PAGE protein bands were transferred by electrophoretic migration to PVDF membrane (PALL Europe Ltd, UK) using a semi-dry transfer cell (Bio-Rad, UK), a technique known as Western blotting. The PVDF membrane was wetted with methanol to reduce its hydrophobicity, then the membrane and the prepared gel were soaked in n-cyclohexyl-3-aminopropanesulphonic acid (CAPS) buffer pH 11.0 (Sigma, UK) for 30 minutes. Filter papers were soaked in the same buffer for 5 minutes. A stack was formed on the anodal plate of the blotting apparatus consisting of, from bottom to top, six filter papers, the PVDF membrane, the SDS-gel slab and three filter papers. After placement of the gel on top of the membrane, a clean Pasteur pipette was gently rolled across the surface to ensure the exclusion of any trapped air and improve contact between the gel and the membrane. The cathodal plate was secured in position and protein transfer was carried out for 2 hours using a current set at 2 mA $cm^{-2}$ of the membrane surface area, up to a maximum of 25 V. After transfer, the gel was stained with Coomassie Brilliant Blue and de-stained to confirm that protein transfer had occurred. The PVDF membrane was stained and de-stained similarly to visualise the transferred protein bands, unless it was to be used for immunostaining.

Immunostaining of SF protein transferred to PVDF membrane was carried out using a polyclonal antibody to *B. amphitrite* SIPC (Matsumura et al., 1998c). Various procedures were tried for immunostaining. Blocking mediums tested were 3% w/v gelatine, 3% w/v dried skimmed milk powder and 1 x Casein solution. Washing regimes included the use of TBS, 1x casein, 0.3 x casein and TTBS. Various concentrations of

primary and secondary antibody and incubation periods were also tested. The method used routinely was as follows. The membrane was rinsed briefly in TBS and blocked for a minimum of 1 hour in 1 x casein solution (Vector Ltd, UK). The membrane was washed for one hour in TBS with renewal of the TBS every 15 minutes. The membrane was incubated in 1:1000 dilution of the primary antibody for a minimum of 1 hour and then washed for 1 hour in TTBS with renewal of the TTBS every 15 minutes. The membrane was incubated in 1:2000 dilution of the secondary antibody, anti-rabbit IgG (Sigma, UK), for 30 minutes and then washed for 45 minutes in TBS with renewal of the TBS each 15 minutes. The membrane was then stained using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue alkaline phosphatase (BCIP/NBT) (Sigma, UK) until active bands became visible. Finally, the membrane was transferred into $dH_2O$ to arrest the colour change reaction before placing on tissue paper to dry.

## 2.4.2 Protein extraction from barnacles

The methods used to extract SF (a protein mix) from adults and subsequent steps through to purification of an SIPC-like protein were a modification of methods described by Matsumura et al. (1998a and b). All extraction procedures were carried out at 4°C or on ice.

### 2.4.2.1 Preparation of SF

Adult barnacles were first cleaned by brushing under running tap water and then drained briefly to remove excess water. Individuals were removed from their substrate, and their weight and volume recorded. The whole barnacles were crushed for 30 minutes in 1.5 volumes of 50 mM Tris-HCl, pH 7.5 using a pestle and mortar. The large shell particles were removed manually and the remaining homogenate stirred for 120 minutes. The liquid was then centrifuged in 50 ml aliquots at 40,000g for 15 minutes to remove particulate matter. The pellet was discarded and the supernatant was filtered through filter paper (Whatman No. 3, Whatman, UK) giving a relatively clear, pale orange-pink

liquid. The volume was recorded and the protein concentration was determined by a total protein assay (2.4.1.1).

The crude extract was stirred while ammonium sulphate, $(NH_4)_2SO_4$ (Sigma, UK), was added slowly to a 70% concentration (472 g $l^{-1}$). The mixture was stirred overnight, then centrifuged in 50 ml aliquots at 40,000g for 15 minutes and the supernatant discarded. The pellet was resuspended in a small volume of 50 mM Tris-HCl, pH 7.5 and placed in dialysis tubing with sufficient space to allow for a 60% volume expansion. The solution was dialysed against 50 mM Tris-HCl, pH 7.5 with two changes of the buffer at 3 hourly intervals, and dialysis continued overnight. The desalted extract was centrifuged at 75,000g for 90 minutes in 10 ml aliquots. The pellet was discarded and the supernatant was filtered through a 0.2 μm low protein binding cellulose acetate membrane (Whatman, UK) giving a clear orange-red liquid. The volume was recorded and the protein concentration was determined by a total protein assay (2.4.1.1). Aliquots of this concentrated crude extract were stored at -80°C for later use in settlement assays and for purification.

### 2.4.2.2 Isolation of SIPC

The SIPC was purified from the crude extract by two stages of low pressure liquid chromatography (ion exchange followed by size exclusion) using an Econopump system with Econosystem fraction collector and chart recorder (Bio-Rad, UK). Active fractions were determined by bioassays and/or immunostaining (using the *B. amphitrite* anti-76 kDa antibody) after each stage. Various parameters were tested to establish workable practices. The standard conditions used in the project are described below.

**Ion exchange chromatography**

Approximately 100 mg total protein of the concentrated crude extract (SF), diluted in 30 ml of sample buffer, 50 mM Tris-HCl pH 7.5, was applied to 1.5 cm diameter x 10 cm chromatography column packed with a cation exchanger, Q-Sepharose (Pharmacia Biotech, UK) equilibrated with 50 mM Tris-HCl, pH 7.5. The buffer was also used for

elution of the sample from the column. An initial elution period, using only the buffer, displaced unattached protein. Adsorbed proteins were then eluted from the column with a 0 - 0.5 M NaCl (Sigma, UK) gradient, over 120 minutes at a rate of 1 ml $min^{-1}$. The column was then washed with 1 M NaCl for 60 minutes, which displaced a further small amount of protein and the column was washed further with buffer until the chromatograph baseline was reached. Fractions of 5 ml were collected. The protein concentration of individual fractions was determined by a total protein assay (2.4.1.1).

**Determination of active ion exchange fractions**

Active ion exchange fractions were determined by a nitrocellulose membrane settlement assay (2.2.2) and/or immunostaining (2.4.1.2.3). A 24-spot assay was prepared with a systematic pattern of protein spots applied across the membrane. A sequential selection of fractions (most frequently all even numbered fractions) was applied to every other well giving a checkerboard effect of protein-adsorbed concave spots and blank concave spots. Larval settlement was recorded at 24- and 48-hour intervals and active fractions were decided by the degree of settlement on the different protein spots. Additionally, active fractions were determined by immunostaining using a polyclonal antibody to the 76 kDa subunit of the *B. amphitrite* SIPC. Bioassay and immunostaining results were compared to the chromatograph, and for later samples active fractions were determined from this printout. Fractions were stored at -80°C for the duration of the bioassay.

Active fractions were pooled and concentrated to ca. 2 ml using an ultrafiltration unit (Amicon, UK) with a YM-30 membrane (Millipore, UK). The sample was then concentrated to a volume of ca. 1 ml by centrifugation at 5,000g in a YM-30 centrifugal filter device (Centricon, Millipore, UK). The protein concentration was determined by a total protein assay (2.4.1.1). The reconcentrated sample was stored at -80ºC unless proceeding immediately to the next step.

**Gel filtration chromatography**

The reconcentrated sample was applied to a 1.5 cm diameter x 100 cm column, packed with a size exclusion medium, Sephacryl S-200 (Pharmacia Biotech, UK), equilibrated

with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5. Approximately 15-20 mg total protein was eluted with the same buffer at a rate of 0.5 ml $min^{-1}$ and 4 ml fractions were collected. The protein concentration was determined by a total protein assay (2.4.1.1). Active fractions were identified by a further nitrocellulose membrane settlement assay (2.2.2) using a systematic pattern of protein spots (as described for the bioassay of ion exchange fractions) and/or immunostaining (2.4.1.2.3). Later samples were identified by reference to the chromatograph. Fractions were stored at -80°C for the duration of the bioassay. The active fractions were pooled and concentrated by centrifugation at 5,000g in a YM-30 centrifugal filter (Centricon, Millipore, UK). The protein concentration was determined by a total protein assay (2.4.1.1). The sample was stored at -80°C for later protein subunit isolation.

**Protein subunit isolation**

The subunit equivalent to the 76 kDa *B. amphitrite* SIPC subunit was selected for isolation by electrophoresis. The subunit was identified by immunostaining (2.4.1.2.3) using a polyclonal antibody to the 76 kDa *B. amphitrite* SIPC subunit. Multiple aliquots of the sample were electrophoresed on 10% standard minigels (2.4.1.2.1). The appropriate band was excised from each gel lane and these bands were pooled.

# 2.5 Molecular biology-related protocols

## 2.5.1 Preparation of complementary DNA (cDNA)

**Isolation of total RNA (TotRNA)**

The shells were removed from ca. 40 adult barnacles and ca. 500 mg of body tissue was ground to a fine powder in liquid nitrogen using a pestle and mortar. The ground tissue was then suspended in liquid nitrogen and transferred to a sterile 50 ml centrifuge tube. For each 100mg of tissue, 1 ml Trizol Reagent (Invitrogen, UK) was added and the RNA was extracted following the manufacturer's protocol. Briefly, the sample was

incubated for 5 minutes at room temperature. For each ml of Trizol, 0.2 ml chloroform was added to the tube and it was shaken vigorously to mix. The tube was centrifuged at 12,000g for 15 minutes at 4°C. The upper phase, containing the RNA, was then removed taking care to ensure no contamination from the boundary layer or the lower phase. The TotRNA was then precipitated by adding 0.5 ml isopropanol $ml^{-1}$ of Trizol used. The mixture was incubated for 10 minutes at room temperature and centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was discarded and the pellet washed with 75% ethanol (1 ml of ethanol $ml^{-1}$ of Trizol). The pellet was dissolved in 150-400 $\mu$l sterile $dH_2O$ by swirling gently. The RNA concentration was measured spectrophotometrically by measuring to $A_{260}$. Excess TotRNA not required for the next stage of preparation was stored at -80°C after the addition of 3 volumes of 100% ethanol.

**Isolation of poly A mRNA from TotRNA**

Poly A mRNA was isolated from 0.25 mg of TotRNA using an Oligotex mini prep kit (Qiagen, UK) following the manufacturer's protocol. The poly A mRNA was eluted in a final volume of 40 $\mu$l $dH_2O$ and the concentration determined spectrophotometrically by measuring to $A_{260}$.

**Synthesis of first strand cDNA**

The synthesis of first strand cDNA was achieved using Superscript II RNase H- Reverse Transcriptase (Invitrogen, UK) or Thermoscript RNase H- Reverse Transcriptase (Invitrogen, UK) in the presence of an Oligo $(dT)_{17}$ or an adapter primer (CGAT17) and dNTPs. In both cases, a 20 $\mu$l synthesis mix containing the mRNA was prepared and incubated following the appropriate product protocol. A total of 1 $\mu$l *E. coli* RNase-H was added to the reaction, which was then incubated at 37°C for 20 minutes to remove the mRNA. The cDNA was stored at -20°C.

## 2.5.2 Amplification of DNA by polymerase chain reaction (PCR)

.

### 2.5.2.1 Standard reaction parameters

Two different thermocylers were used for PCR amplifications (PTC-100, MJ Research, USA; PTC-0200 MJ Research, USA). PCR reactions were set up with volumes of between 10 and 100 $\mu$l. Most PCR reactions used Taq DNA polymerase (Invitrogen, UK). However, for PCR products to be sequenced, the proof-reading polymerase, Pfu (Stratagene, UK) with exonuclease activity, was used. All PCR reactions were prepared on ice.

A 50 $\mu$l standard reaction comprised:-

| | |
|---|---|
| 1-2 $\mu$l | template DNA |
| 5 $\mu$l | 10 x PCR buffer |
| 1.5 $\mu$l | 50 mM (i.e. 1.5 mM final concentration) $MgCl_2$ |
| 1 $\mu$l | 10 pmol forward primer |
| 1 $\mu$l | 10 pmol reverse primer |
| 0.5 $\mu$l | Taq DNA polymerase |
| | + sterile $dH_20$ to 50 $\mu$l |

The reaction parameters were altered to meet Pfu requirements i.e. the primer concentration was increased 3-fold, and 1 $\mu$l of Pfu was used in a standard 50 $\mu$l reaction. Primer concentrations were also altered for certain cDNA amplifications using Taq DNA polymerase, i.e. degenerate primers were used at a concentration of 1 $\mu$g per 50 $\mu$l reaction.

A stock mix, excluding the template DNA and Taq DNA polymerase, was prepared in a 1.5 $\mu$l microfuge tube and vortexed briefly to mix. The Taq DNA polymerase was then added, the tube contents mixed gently and then centrifuged briefly. Aliquots of the stock mix were transferred to PCR reaction tubes and the template DNA was added inside the reaction tube lid. The tube was then sealed and centrifuged briefly to combine reagents.

When the PTC-100 thermocycler (i.e. without a heated lid) was used, an overlay of mineral oil was added to each reaction tube to prevent evaporation.

### 2.5.2.2 PCR thermocycling

A total of 25 – 30 thermal cycles were performed using the following temperature profile:-

| Step | Temperature | Duration | |
|---|---|---|---|
| | | 0.5 $\mu$l reaction tubes | 0.2 $\mu$l thin wall reaction tubes |
| Initial denaturation | 94°C | 2 minutes | 1 minute |
| Reaction cycle (repeated 25-30x) : | | | |
| Denaturation | 94°C | 1 minute | 30 seconds |
| Primer annealing | Tm of primer – 5°C | 1 minute | 30 seconds |
| Primer extension | 72°C | 1 minute 1000 $bp^{-1}$ | 1 minute 1000 $bp^{-1}$ |
| Final extension | 72°C | up to 10 minutes | up to 10 minutes |
| Holding conditions | 8°C | until removed from thermocycler | until removed from thermocycler |

### 2.5.2.3 Design of oligonucleotide primers for efficient PCR reactions

Whenever possible, primers were designed using the following guidelines to optimise efficiency:- 1) 18-24 nucleotides in length; 2) 45-55% G or C bases; 3) a melting temperature (Tm) of ca. 60°C, with similar Tms between primer pairs. The Tm was calculated roughly by attributing 2°C for each A or T base and 4°C for each G or C base (Thein and Wallace, 1986); 4) no self-complementarity, i.e. a sequence of bases that would find a match within the primer, both within a single primer and between a primer

pair; and 5) no 3' complementarity between primers, in order to reduce primer-dimer artefacts that reduce the yield of the desired product (Innis and Gelfand, 1990).

### 2.5.2.4 Optimising PCR reactions

Certain PCR amplifications proved difficult. Thus, at times, the standard reaction protocol was amended to attempt to improve PCR efficiency. Measures that were taken were:- 1) changes to temperature of the thermocycling annealing phase; 2) alternative buffer system and source of magnesium ions i.e. PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl (Invitrogen, UK) with magnesium chloride, or PCRx Amplification Buffer (Invitrogen, UK) with magnesium sulphate; 3) variation in the concentration of Taq DNA polymerase from 0.1 $\mu$l (0.5 U) to 0.5 $\mu$l (2.5 U) in a 50 $\mu$l reaction; 4) inclusion of a reaction enhancer (PCRx Enhancer System, Invitrogen, UK) between 0.5x and 2x concentration, with appropriate changes to PCR thermocycling temperatures; 5) variation in concentration of magnesium ions in the reaction mix from 0.5 mM to 2.5 mM concentration, and 1 mM to 3 mM when used with the reaction enhancer; 6) changes to the duration of the thermocycling extension phase (Innis and Gelfand, 1990; www.invitrogen.com).

### 2.5.2.5 Visualisation of DNA and PCR products

Horizontal agarose gel electrophoresis (1-3%) was used to visualise DNA and PCR products using TAE buffer (0.4 M Tris; 0.1% glacial acetic acid; 0.001 M NaEDTA pH 8.0) with 0.015 mg $ml^{-1}$ ethidium bromide incorporated into the gel. Gels were prepared by melting the agarose in TAE. A comb was placed towards the top edge of the plate and removed when the gel had set to form wells into which the sample was applied. A molecular weight (MW) ladder was placed in the first lane of all gels. Each sample was prepared for electrophoresis by adding loading buffer (20% glycerol; 0.1 M NaEDTA pH 8.0; 1% SDS; 0.25% bromophenol blue; 0.25% xylene cyanol) to a final concentration of 10%. Electrophoresis was carried out at 5 V $cm^{-1}$ distance between electrodes for all fragments > 100 bp. Products < 100 bp were run at a lower voltage to

improve resolution. Products were visualised under UV light and their size estimated by comparison to the MW ladder.

## 2.5.3 Clean-up and precipitation of PCR products

### 2.5.3.1 Phenol/chloroform clean-up of PCR products

All procedures were carried out in a ventilated fume cupboard. The PCR product was transferred to a clean 1.5 ml microfuge tube and an equal volume of a phenol: chloroform: isoamyl alcohol, 25:24:1, mixture was added and mixed by inversion. The tube was centrifuged at 12,000g for 10 minutes. The upper phase was removed by pipette, taking care not to disturb the phenol/chloroform lower phase or boundary layer, and transferred to a clean 1.5 ml microfuge tube. The phenol/chloroform step was repeated and the cleaned PCR product was precipitated (2.5.3.2).

### 2.5.3.2 Precipitation of PCR products

PCR products were precipitated by two methods; sodium acetate/ethanol precipitation and ammonium acetate/isopropanol precipitation. The PCR product was transferred to a clean 1.5 ml microfuge tube and precipitated with, either 0.1 volume 3 M sodium acetate and 2.5 volume 99.5% ethanol, or 0.5 volume 7.5 M ammonium acetate and 1.5 volume of isopropanol. The reagents were mixed well by inversion and then centrifuged at 12,000g for 30 minutes. The supernatant was removed and the pellet was washed with 200 $\mu$l of 70% ethanol, and centrifuged at 12,000g for 15 minutes. The supernatant was removed and the pellet air dried for 10 minutes at room temperature.

## 2.5.4 DNA Cloning and product sequencing

### PCR product preparation

The products of two 50 $\mu$l PCR reactions (2.5.2.1) were precipitated by the ammonium acetate/isopropanol method (2.5.3.2). The product was resuspended in 10-15 $\mu$l of sterile $dH_2O$ and visualised on a 1% TAE agarose gel (2.5.2.5). An appropriate sized band was excised using a clean razor blade ensuring the time under UV light was minimised. The PCR product was removed from the gel slice by centrifugation through a glass wool column. To prepare the column, a tuft of glass wool was packed into a 0.5 ml microfuge tube that had been pierced through the cap and base with a hot needle. The gel slice was placed on top of the glass wool and the tube closed. This tube was then placed inside a 1.5 ml microfuge tube with its cap removed. The device was centrifuged at 6,000 rpm for 10 minutes. The DNA passed through the glass wool and was retained in the bottom of the larger tube. The volume was measured, adjusted with sterile $dH_2O$ to a volume of 100 $\mu$l. The product was then precipitated using ammonium acetate (2.5.3.2), and resuspended in sterile $dH_20$. Where the original PCR product was prepared using Taq polymerase, the gel-purified product was 'end-polished' using Pfu DNA polymerase (Stratagene, UK) and a 10 $\mu$l reaction was prepared on ice as follows:-

| | |
|---|---|
| Gel-purified product | 7.5 $\mu$l |
| 10 x Pfu buffer | 1.0 $\mu$l |
| 10 mM dNTPs | 1.0 $\mu$l |
| Pfu DNA polymerase | 0.5 $\mu$l |

The reaction was incubated in the thermocycler for 30 minutes at 72°C. This step was not required when the original PCR product was prepared using Pfu. The PCR products were blunt-end cloned into pBluescript (Stratagene, UK) that had been cleaved by the restriction enzyme EcoR V using T4 DNA ligase.

**DNA ligation into vector DNA**

A 10 $\mu$l ligation reaction was prepared on ice as follows:-

| | |
|---|---|
| PCR product | 1-5 $\mu$l |
| 10 x Ligase buffer | 1 $\mu$l |
| pBluescript KS-(EcoR V) | 1-2 $\mu$l |
| T4 DNA ligase | 1 $\mu$l |
| Sterile $dH_2O$ | to 10 $\mu$l |

The mix was ligated overnight at 16°C resulting in recombinant DNA molecules, i.e. the cloning vector with an inserted DNA fragment. The reaction was terminated by heat kill at 65°C for 10 minutes.

**Preparation of Luria-Bertani (LB)/Agar plates and transformation**

Luria-Bertani (LB)/Agar plates were prepared in advance. A total of 100 ml of LB Agar (LB media , i.e. 1 g Bacto-tryptone; 0.5 g Bacto-yeast; 1 g sodium chloride, with 1.5 g Bacto-agar in 100 ml $dH_20$ and sterilised by autoclaving) was dissolved by microwaving on half power for 3-5 minutes. The LB Agar was allowed to cool to ca. 45°C. A 100 $\mu$l aliquot of 100 mg $ml^{-1}$ ampicillin was added and swirled to mix. Aliquots of 10-12 ml of the ampicillin-treated LB Agar were poured onto culture plates and the plate swirled gently to ensure complete coverage. The surface of the LB Agar was flamed briefly to remove bubbles. The culture plates were dried upside down and slightly open at 37°C for 1 hour. The lid was replaced and the plates were stored upside down at 4°C in a sealed polythene bag until required.

Prior to transformation, 4 $\mu$l of isopropyl-ß-D-thiogalactopyranoside (IPTG) stock (28 mg $ml^{-1}$ in $dH_20$) and 50 $\mu$l of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) stock (50 mg $ml^{-1}$ in N-N-dimethylformamide) were added to each plate and spread evenly across the surface using a glass spreader. The lid was replaced immediately and the culture plates were dried upside down at 37°C for 1 hour.

**Bacteria transformation**

A total of 3 $\mu$l of the ligation reaction was added to 100 $\mu$l of chemically competent XL1- blue cells thawed on ice. The transformation reaction was mixed gently and was incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds, then placed immediately on ice for 2 minutes. The cells were added to 400 $\mu$l of LB media and incubated while shaken at 37°C for 1 hour. A 100 $\mu$l aliquot of the media/cell mix was transferred to each plate and spread evenly across the surface of the LB Agar plate using a sterile glass spreader. The lid was replaced immediately after spreading, and the culture plates were incubated upside down at 37°C overnight.

**Screening clones**

Positive transformants were identified by blue - white selection, with white indicating that the cell may contain the recombinant DNA. White colonies were picked onto a further agar plate, with a grid drawn externally and each square numbered, so that each colony was confined to one square of the grid. The new plate with the 'spotted' colonies was incubated upside down at 37°C overnight. Positive colonies were screened by PCR in 10 $\mu$l reactions using T3 and T7 plasmid-specific primers. White bacterial colonies were picked using a sterile pipette tip and added to the PCR reaction. The PCR reactions followed the thermal profile given in Section 2.5.2.2 for 25 cycles annealing at 54°C with an appropriate extension time. Colonies with the correct size insert were determined by electrophoresis on a 1% TAE Agarose gel (2.5.2.5).

**Preparation for sequencing**

Colonies selected for sequencing of the DNA insert were picked into a covered test tube containing 5 ml of LB media with 1 $\mu$l $ml^{-1}$ ampicillin. The culture was incubated while shaken at 37°C overnight. A total of 3 ml of the medium (2 x 1.5 ml microfuge tubes) was centrifuged at 6,000g for 2 minutes, the supernatant was removed and the pellets from the two tubes were combined. Plasmid DNA was isolated from the cells using a Plasmid Miniprep Kit (Qiagen, UK) following the product protocol. The plasmid DNA was precipitated using ammonium acetate (2.5.3.2). The sample was sequenced commercially (MWG Biotech, UK).

# Chapter 3

# Laboratory culture of *Elminius modestus* and *Semibalanus balanoides*

## 3.1 Introduction

The success of the routine use of a barnacle species in the laboratory is dependent on the successful culturing of sufficient competent larvae to meet experimental demands. Additionally, to avoid repeated shore collections of adults and to be independent of seasonal availability of larvae, a laboratory broodstock is highly desirable. The species *E. modestus* and *S. balanoides* were selected for experimentation. Adult and larval culture of *E. modestus* has been achieved previously (Moyse, 1960; Barker, 1976; Billinghurst et al., 2001). The culture of *S. balanoides* is more challenging, as it has an annual cycle to larval production, which may be difficult to be overcome by a simple temperature and light regime manipulation. The aim of this research was to define workable practices for adult maintenance and larval culture that would ensure an adequate supply of cypris larvae for laboratory settlement behaviour experiments. Larval culture only was attempted for *S. balanoides*.

## 3.2 Materials and methods

### 3.2.1 *Elminius modestus*

**General considerations**

Using published parameters as guidance (e.g. Moyse 1960, Barker, 1976; Billinghurst et al., 2001), combined with laboratory knowledge of the successful culture of *B. amphitrite*, a regime was established for the culture of *E. modestus* adults and larvae. During trials, the key variable for both adult and larval cultures was temperature. Other parameters, i.e. light:dark (L: D) cycles, water renewal and larval concentration, were also varied for larval culture. The standard conditions used in the project are described below.

**Adult maintenance**

Early attempts at adult culture used *E. modestus* individuals that had been held in an ambient temperature aquarium for ca. 4 months. A replacement brood stock was established using adults collected from Great Bull Hill, Exmouth, Devon, United Kingdom (50°36.9'N 3°25.8'W) during December 2001. Further replacements were collected during March 2002 and October 2002. There is a large population of *M. edulis* with *E. modestus* attached to the shells at Great Bull Hill, which enabled adult barnacles to be collected and transported easily. Where possible empty mussel shells were collected; otherwise live *M. edulis* with barnacles attached were collected and shucked. The shells were then fixed onto Perspex plates (10 cm x 25 cm) using an aquarium silicone sealant (Rhodia Sealants Ltd, Leicester, UK) (Figure 3.1a). Six plates, with ca. 2,000 individuals in total, were housed in two 20-litre tanks containing aerated seawater at a room temperature of 20±1°C (Figure 3.2). Seawater, collected from the northeast coast of England, UV treated and filtered through a 10 μm filter, was changed daily or every two days. At each water change, the adults were cleaned by brushing gently under running tap water (Figure 3.1b), before returning to the aquarium. The broodstock was maintained on a 16:8 L: D cycle and fed daily on newly hatched *Artemia* sp. (Sanders, Great Salt Lake *Artemia* cysts). Additionally, a reserve broodstock was held in an ambient temperature aquarium with a constant-flow re-circulating artificial seawater supply.

**Larval culture**

The adults were encouraged to synchronise larval release by overnight emersion. A point light source, external to the tank and close to the water surface, was used to aggregate positively phototactic nauplii that were released by adults. The larvae were collected by pipette and transferred into a holding beaker containing filtered seawater and a small amount of the alga *Skeletonema costatum*. Larval release varied between 1,000 to 20,000 individuals, and releases of less than 5,000 were discarded. Larvae were cultured in 0.7 μm filtered seawater treated with antibiotics (21.9 mg $l^{-1}$ penicillin G and 36.5 mg $l^{-1}$ streptomycin, Sigma, UK) at a concentration of 1 larva $ml^{-1}$ and a maximum batch size of 10 thousand individuals. The culture was maintained in an incubator at

Figure 3.1a: Perspex plate attached with *E. modestus* broodstock

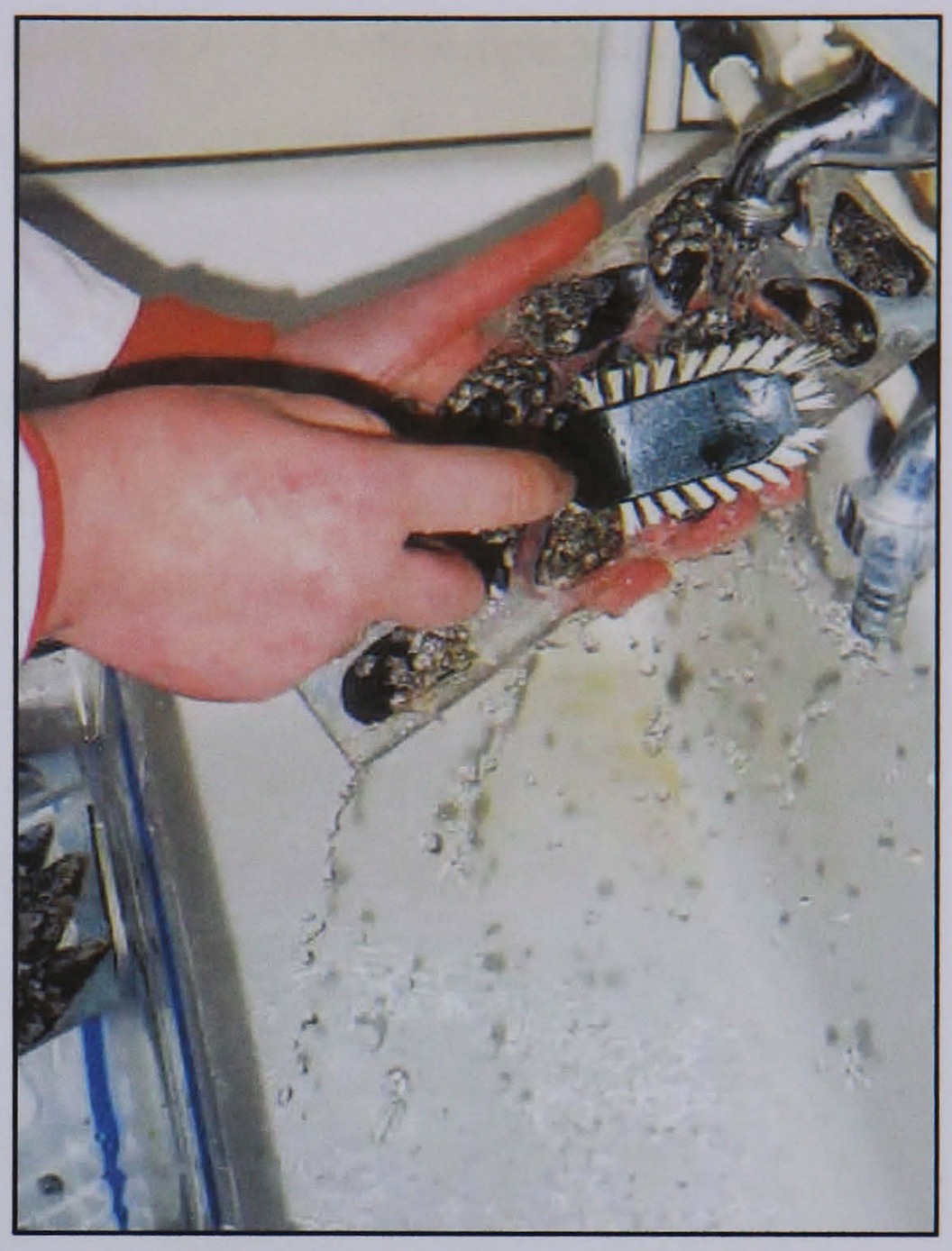


Figure 3.1b: Cleaning of broodstock under running tap water.

Figure 3.2: Broodstock attached to Perspex plates housed in two 20 l aquaria at 20±1°C.

22±1°C on a 16:8 L: D cycle with the water aerated gently at room temperature using a small pump. The nauplii were fed *S. costatum* at a concentration of ca. 1 x $10^5$ cells $ml^{-1}$.

With day 0 used to describe the date of release, the water was changed on the fourth day and the *E. modestus* larvae reached the cyprid stage on the seventh, or occasionally the eighth day. If the culture quality deteriorated, as a result of precipitation of algal cells to the bottom of the culture vessel, a further water change was carried out on day 6 to avoid the entanglement of cyprids in flocculent material. On each occasion larvae were removed from the culture by filtering through a series of filters (300 μm, 250 μm, and 160 μm). All Day 4 larvae passed through the 300 μm filter with the majority of them retained by the 250 μm. Small larvae, i.e. those that were retained by the 160 μm filter, were removed from the culture and generally accounted for a ca. 10% loss of larvae. The culture was terminated when 70-80% of larvae had reached the cyprid stage, and larvae were removed from the culture by filtering as described previously. The cypris larvae were retained by the 250 μm filter, while the majority of remaining nauplii were retained by the 300 μm filter. The cyprids were washed from the filter into a crystallising dish using 0.45 μm filtered seawater. The dish was swirled gently to concentrate the cyprids in the centre of the dish. The cyprids were removed using a pipette, leaving behind as much of the unwanted debris (including moults, nauplii and algal particles) as possible. The collected cyprids were placed in another clean crystallising dish containing 0.45 $\mu$m filtered seawater. The culture was cleaned in this way several times. The dish was covered with Parafilm, which was then perforated, and the dish was stored at 6°C. Day 0 cyprids were used immediately for settlement assays, or were stored in the crystallising dish at 6°C for later use. On one occasion cyprids were stored at 22±1°C (to investigate the effect of holding temperature on cyprid behaviour in assays) and were held within plankton netting in a covered plastic container containing gently aerated seawater.

## 3.2.2 *Semibalanus balanoides*

### Larval culture

Adult *S. balanoides*, attached to *Mytilus* valves, were collected on April 18th 2002 from Blackhall Rocks, Co. Durham (54°44.5'N 1°16.1'E) and were used immediately for the release of cypris larvae. About 500 individuals were removed from their substratum. They were gently crushed, placed at one end of shallow rectangular glass container (20 cm x 40 cm x 5 cm) and then covered with 0.45 $\mu$m filtered seawater. A point light source was placed at the opposite end of the container and aluminium foil was placed over the vessel to exclude light. Larvae were released from the crushed tissue and, being photopositive, they congregated near the light source, thereby facilitating their collection. Ca. 10,000 larvae were transferred into a holding beaker containing a small amount of *S. costatum* in filtered seawater. The larvae were cultured as described for *E. modestus*, but at a temperature of 11±1°C on a 16:8 L:D cycle. Water changes were carried out every seven days, and the numbers of living larvae returned to the culture recorded. A few larvae reached the cyprid stage after 25 days, and were removed from the culture and their condition noted.

### Collection of wild *Semibalanus balanoides* cypris larvae

Due to the failure of the laboratory culture of *S. balanoides* larvae, wild cyprids were collected for laboratory assays. Wild *S. balanoides* cyprids were collected from Seaham, Co. Durham (54°20.8'N 2°48.5'W) on May 3rd, 15th and 24th 2002. Larvae were collected by towing a 250 $\mu$m plankton net by hand alongside the harbour wall. The larvae collected in a removable filter at the end of the net and were washed into small containers. In the laboratory, the cyprids were transferred into a crystallising dish and the sample was 'cleaned' as described for *E. modestus*. The crystallising dish was covered with Parafilm and perforated before being retained overnight in a refrigerator at 6°C.

## 3.3 Results

### Adult maintenance and larval culture of *Elminius modestus*

First attempts at *E. modestus* adult and larval culture were unsuccessful. Adults were maintained at a room temperature of 18±1°C. Additionally, as the room was illuminated continuously, a dark phase was achieved by covering the tanks overnight, resulting in an 8:16 L: D cycle. High adult mortality (>50%) was observed within weeks and larval release was slow. Larval cultures, carried out at 18±1°C, had a mortality of ca. 90-95% with those larvae reaching the cyprid appearing unhealthy. There was also a high incidence of incomplete ecdysis. A replacement broodstock (December 2001) was maintained at a room temperature of 20±1°C with a controlled 16:8 L: D cycle. The broodstock remained healthy, and larval releases were good. However, larval culture remained unsuccessful at 18±1°C. The culture temperature was increased to 25±1°C and healthy cyprids developed in 5 days, but mortality was high (ca. 80%). The temperature was reduced several degrees, increasing the culture period, and high mortality continued. A water change mid-culture was introduced and a temperature of 22±1°C became routine. Cyprids developed in 7 days with larval survival commonly ca. 60%, and always within the range of 40-80%.

Figure 3.3 illustrates each stage of the development of *E. modestus* larvae and the visible features that aided identification are described. Setation and appendage detail, that would have enabled absolute identification (Knight-Jones and Waugh, 1949; Barker, 1976), cannot be discerned from the photographs. As a random selection of individuals was photographed on each occasion, the images do not illustrate the development of a single larva and thus ontogenetic size changes cannot be clearly discerned from this series.

### Larval culture of *Semibalanus balanoides*

The culture of *S. balanoides* larvae was unsuccessful. Table 3.1 is a record of surviving larvae at each water change and at the end of the culture, with notes on the condition of


Figure 3.3: Larval development of the species *Elminius modestus.* Key visible identification features (from Knight-Jones and Waugh, 1949):- (a) Stage I – front-lateral horns pointing obliquely backwards (i.e. not visible), (b) Stage II – characteristically slender, (c) Stage III - similar length to Stage II, but stouter shape and (d) Stage IV – development of a rounder shape.

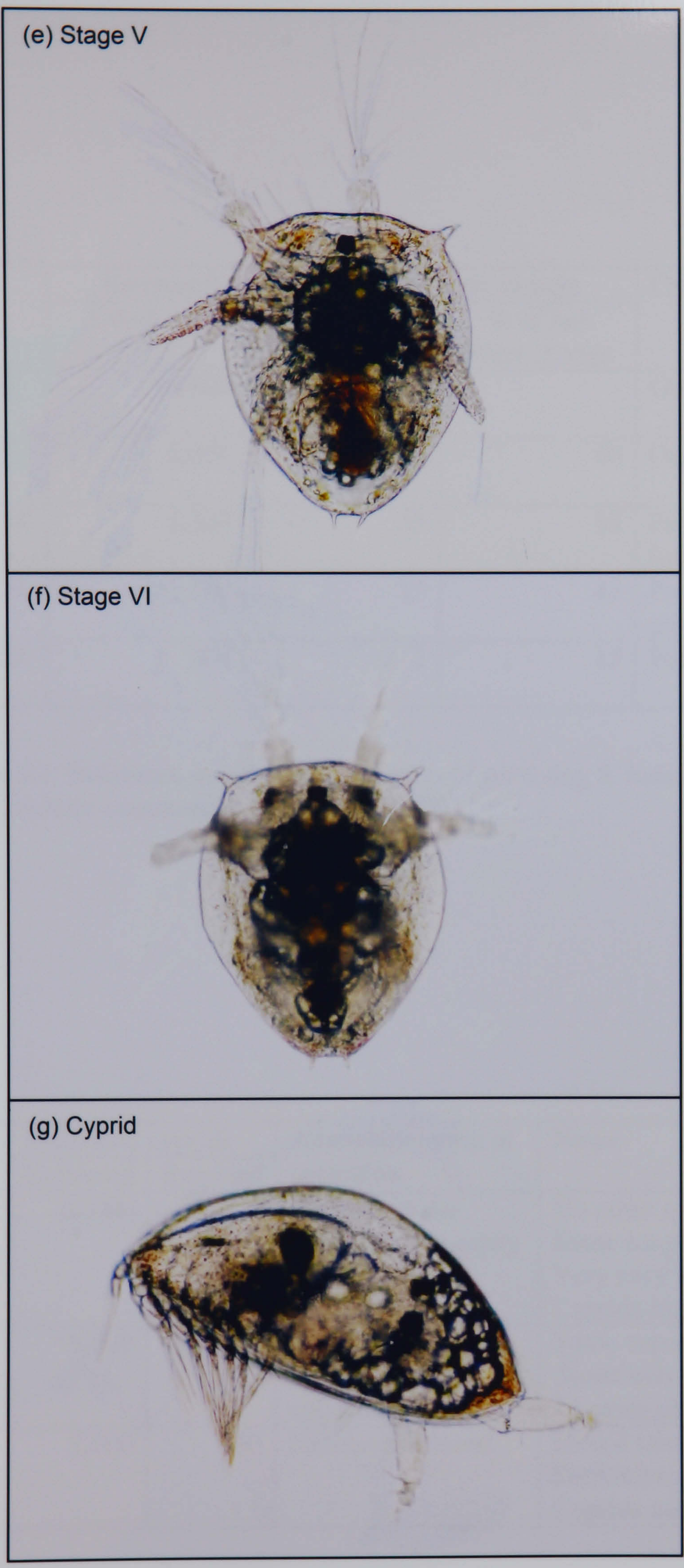



Figure 3.3 (continued): Larval development of the species *Elminius modestus.* Key identification features (from Knight-Jones and Waugh, 1949:- (e) Stage V –shield-like shape, (f) Stage VI – three black eye spots, (g) Cyprid – highly distinctive shape.

| Day | Estimated survivors at each water change | | | Condition of larvae |
|---|---|---|---|---|
| | Count (n) | % of initial culture | % of last water change | |
| Day 0 | 10,000 | 100 | - | Good |
| Day 7 | 6,000 | 60 | 60 | Good |
| Day 14 | 3,500 | 35 | 58 | Fair. Some evidence of a fungal growth |
| Day 21 | 1,500 | 15 | 43 | Poor. Increased fungal growth. |
| Day 25 | 200 | 2 | 13 | Poor. Moribund cyprids. |

Table 3.1: Estimated number and condition of surviving *S. balanoides* larvae of the larval culture commenced on April 18th 2002.

| Date | Estimated cyprids | | Availability/ease of collection | Notes |
|---|---|---|---|---|
| | Collected | Retained | | |
| May 3rd | 10,000 | 9,750 | Dense in water column. Very easily collected. | No other species present.<br>Some nauplii present.<br>Very easy to clean sample.<br>Cyprids highly phototactic. |
| May 15th | 8,000 | 7,750 | Dense in water column. Easily collected. | Some copepods also present.<br>Relatively easy to clean sample.<br>Cyprids phototactic. |
| May 24th | 2,000 | 500 | Numbers reduced. | Mixed sample.<br>Difficult to clean sample.<br>Cyprids not phototactic. |

Table 3.2: Record of collection of *S. balanoides* cyprids from Seaham Harbour, Co. Durham, UK (54°50.1N 1°20.3E) during May 2002.

the larvae. At days 7 and 14 of the culture, only ca. 60% survived compared to 7 days earlier. Mortality increased after day 14, with 43% of the day 14 culture surviving at day 21. Only 13% of these, which was 2% of the original culture, reached the cyprid stage 4 days later at day 25. As it was too late in the season for a further attempt at laboratory culture, wild cyprids were collected.

**Collection of wild *Semibalanus balanoides* larvae**

Table 3.2 is a record of the number of *S. balanoides* larvae collected during May 2002 with notes recorded at that time. Cyprids were readily available in the water column at the first two collection dates. Numbers had reduced by late May and only a small number of larvae were collected. Additionally, it was difficult to separate the larvae from the final sample because of the presence of other species. Cyprids collected on May 3rd and May 15th were readily phototactic, while those collected on May 24th 2002 were not.

## 3.4 Discussion

***Elminius modestus***

Few publications describe the adult culture of *E. modestus*. In earlier research a broodstock was generally not maintained, it being common practice to use freshly collected adults to release larvae (e.g. Wisely, 1960), or else hatch larvae from removed mature ovigerous lamellae (e.g. Barker, 1976). The unsuccessful adult culture at 18±1°C may have been due to the light regime rather than the temperature. Crisp and Davies (1955) observed that breeding by *E. modestus* occurred providing the temperature remained above 6°C and the food supply was adequate. However, the reserve broodstock, maintained in an ambient aquarium above 6°C, produced few larvae, though periodic system failures, as well as the use of artificial seawater, may have been responsible. The adults appeared to thrive at 20±1°C and they released large numbers of larvae frequently. However, when the room temperature was increased by 2-3°C, with little consequence to the similarly-housed *B. amphitrite* adults, the condition of the

broodstock deteriorated rapidly, indicating that the species was towards the upper limit of its thermo-tolerance.

Previous laboratory culture of *E. modestus* larvae has been achieved under a variety of conditions. The diatom *S. costatum* has long been a preferred food source for many of the Balanidae (e.g. Moyse, 1960; Moyse and Knight-Jones 1967; Barker, 1976). Little has been reported on L: D cycles, while larval density and temperature have been variable parameters. Wisely (1960) did not culture *E. modestus* successfully, with survival of the original culture to cyprid often as low as ca. 0.1%. His lack of success has been attributed to his choice of food (*Phaeodactylum tricornutum* supplemented with dried liver powder) (Barker, 1976), though factors such as larval density (as high as 47 nauplii per ml), combined with the lack of antibiotics in the culture medium and the limited temperature control (room temperature of 17-23°C) would seem additional factors (pers. obs.). Barker (1976) tested three temperatures, 20, 25 and 30°C, though development to the cyprid was achieved only at 20°C. However, larvae from a Japanese broodstock were cultured successfully at 25°C (K. Matsumura pers. comm.; Atienzar et al., 2002), although mortality was not reported. Atienzar et al. (2002) cultured in small volumes with an 8:16 L: D cycle. Replicate parameters were not attempted for this research, as small cultures were impractical for the quantity of larvae required. Larval culture was successful at 18 - 20°C using adults obtained from the same location as this project (Billinghurst et al., 2001), although again, culture volumes were small and mortality was not reported. In the present study, survival was poor at 19°C, but may reflect the use of adults held for some months in unsustainable conditions, such that newly-released nauplii may have had a reduced fitness. Moyse (1960) describes the successful larval culture of *E. modestus* in non-aerated seawater without antibiotics in 'Pyrex' beakers placed towards north facing windows in the laboratory and without temperature control. He recorded a 90% survival of the original culture through to cyprid, which was much greater than current achievements (frequently ca. 60%). Again culture volumes were small and larvae were obtained by dissecting adults. It appears that adults were collected frequently and that the condition of adults would seem to have a direct impact on the success of larval cultures.

Accepting the possible reduced fitness of larvae at the point of release from laboratory-held adults, critical factors for culture success were temperature and water condition. Larvae developed more quickly at higher temperatures, while at lower temperatures with longer development, bacterial contamination and fungal growth were problematic. The culture temperature of 22±1°C, with a water change mid-culture, gave acceptable results, although survival rates were variable.

***Semibalanus balanoides***

Adult *S. balanoides* maintenance was not attempted. The species is an annual brooder, with a short larval release period (3 - 5 weeks) promoted by the spring algal bloom (Crisp, 1956). Clare and Matsumura (2000) suggested that simple laboratory manipulation of conditions could extend the release period of adult culture, as the release is related to food availability, and therefore in turn to temperature and light. Additionally, Massachusetts' coasts (US) experience an extended *S. balanoides* larval supply of >5 months (Pineda et al., 2002). This suggests a naturally long reproductive season and supports the possibility of extending the release period in the laboratory. However, the authors could not rule out the possibility of larval advection from other regions where the barnacles have different reproductive schedules. However, the priority here was to investigate whether larvae could be cultured and used successfully in larval assays. If larval culture becomes routine, broodstock experimentation may be useful.

The unsuccessful larval culture of *S. balanoides* may have been due to the long culture period and resultant fungal growth. Some larvae did survive to the cyprid stage, though they were moribund. A weekly water change was selected to limit the number of times nauplii were filtered, in order to limit potential damage to appendages and setae. While some mortality may have been linked to physical damage, the problem of fungal growth appeared the dominant factor and the culture may have benefited from more frequent water changes, for example every 4 to 5 days. Additionally, although the culture temperature reflected ambient coastal temperatures, it was questioned whether 12°C was ideal. Assays showed that the highest rate of settlement by wild *S. balanoides* cyprids occurred at 19°C (4.3.2). In comparison, peak *E. modestus* settlement occurred at the

same temperature as that of the larval culture (4.3.1), and *S. balanoides* larval culture at their optimum assay temperature should be tested.

**Wild *Semibalanus balanoides* larvae**

The larval collections undertaken in 2002 suggested that the larval supply lasted little more than 3 weeks; it was a short season, but not unusually so, as annual variation in cyprid release and survival may account for the limited supply (Kendall et al., 1985; Todd, 2003). The presence of nauplii in the first sample on May 3rd 2002, combined with the absence of recruitment on the adjacent Featherbed Rocks, suggested that the first collection was at the start of the season. The decline of cyprid availability by May 24th suggested that the supply was nearing completion. Barnacle cyprids remain photopositive up to and including the time of settlement (Crisp and Barnes et al., 1956), such that the decline in phototactic behaviour between collections suggests that larvae collected later in the season may have been more inclined to settle, and it was unfortunate that the quantity collected on May 24th 2002 was inadequate to carry out further assays.

## 3.5 Conclusion

*E. modestus* adult maintenance and larval culture were both routine. Sufficient larvae were cultured to enable extensive experiments using a single cohort. *S. balanoides* larval culture was unsuccessful, though some cyprids were obtained, and alterations to temperature and water changing could improve results. Wild *S. balanoides* larvae were collected easily, though the availability was of short duration.

# Chapter 4

# In search of a temperate model species

# 4.1 Introduction

Early laboratory experiments on barnacle settlement were carried out using temperate species, such as *E. modestus* and *S. balanoides*, although in recent years *B. amphitrite*, a tropical/ semi-tropical species has been favoured due to its prolific nature, adaptability to laboratory conditions, short larval culture period and economic importance as a fouling species (Clare and Matsumura, 2000). Additionally, experimental design has evolved with the introduction of alternative assay methods, such as 24-well plate and nitrocellulose membrane assays. Thus, while our understanding of settlement behaviour is underpinned by that of temperate-species, a comparatively detailed understanding of *B. amphitrite* settlement, based on mechanistic studies, has been attained in recent years. Many early experiments were carried out using wild cyprids of an unknown age and the opportunity remains to reinvestigate temperate species using laboratory-cultured larvae and revised methods, which would enable direct comparisons to *B. amphitrite*. *E. modestus*, an Antipodean fouling species now common around the UK (Crisp and Chipperfield, 1948; Lawson et al., 2004; Muxagata et al., 2004), and *S. balanoides*, a native boreo-arctic species found on both sides of the north Atlantic within the latitudinal range from the artic to Northern Spain and Portugal (Barnes 1957b, 1958; Southward et al., 1995; Jenkins et al., 2000) were selected. The aim of this research was to examine the utility of *E. modestus* and *S. balanoides* as temperate model species for settlement studies using recently developed assay methods.

**Experimental objectives**

A. To investigate whether *E. modestus* and *S. balanoides* cyprids can be used successfully in 24-well plate and nitrocellulose assays.
B. To determine an appropriate temperature and concentration of SF to demonstrate gregarious settlement for *E. modestus* and *S. balanoides* cyprids.
C. To determine the effects of ageing on *E. modestus* cyprid settlement.

# 4.2 Materials and methods

## 4.2.1 *Elminius modestus*

### Experimental design

Experiments were undertaken to meet Objective B (appropriate temperature and concentration of SF) using 24-well plate assays (2.2.1). Day 0 and Day 1 cyprids were selected for the first experiments following trial settlement plates. Objective A (suitability of assay methods) was to be achieved by a review of these results and their analysis. Many cyprids floated in the first experiments and it was decided to carry out specific experiments, within the scope of Objective A, to determine whether the assay could be adapted to reduce cyprid floating. Then, experiments to meet Objective C (the effect of cyprid ageing on settlement) using 24-well plate assays were undertaken. Finally, nitrocellulose membrane assays (2.2.2) were carried out.

### Optimum temperature and concentration of conspecific Settlement Factor (SF)

**Experiment EM1 (Temperature and concentration of SF)**

Two separate experiments were carried out using the 24-well plate assay (2.2.1). Day 0 cyprids from a single cohort were used in assays at six temperatures, 5, 11, 15, 19, 22 and 27°C. Day 1 cyprids from the same cohort were used in assays at four temperatures, 15, 19, 22 and 27°C. For each cyprid age and temperature, four 24-well plates were used; four concentrations, 10, 20, 30 and 40 $\mu$g ml$^{-1}$, of conspecific SF and BSA (as a negative control) were tested with 8 replicates of each treatment. A second negative control of 0.45$\mu$m filtered seawater was used with 8 replicates per assay plate. Cyprid behaviour was observed at 24±2 hour intervals for a period of 72 hours. Assays at 19, 22 and 27°C were repeated using a further larval cohort.

**Experiment EM2 (Concentration of SF)**

Day 0 cyprids were used in the 24-well plate assays (2.2.1) at 22°C. Six concentrations, 0, 0.01, 0.1, 1, 10, 50, 100 and 150 $\mu$g ml$^{-1}$, of conspecific SF were tested with 6 replicates of each treatment. Cyprid behaviour was observed after 24 hours. Assays were repeated using a further larval cohort.

**Attempts to reduce the incidence of cyprid floating**

Experiments EM3 to EM7 were undertaken to investigate whether a simple action could reduce the incidence of cyprids floating in 24-well assays. Unless stated, all experiments were carried out at 22°C. The assays were carried out using 24-well plates (2.2.1), except for Experiment EM6 when alternative assay materials were tested.

**Experiment EM3 (Direct action on floaters)**

The experiment considered if direct action on floaters during the assay would alter cyprid behaviour. Day 0 cyprids were used in one 24-well plate assay with all wells containing 0.45 $\mu$m filtered seawater. Cyprid behaviour was recorded at 24±2-hour intervals for 72 hours. At each 24-hour observation, floating cyprids in 12 wells were coerced to sink by dropping water (from the well) onto them using a Pasteur pipette. The assay was repeated using a further cohort of cyprids

**Experiment EM4 (Different holding temperature and light)**

The experiment considered if different holding temperatures for cyprids and/or different light conditions in assays would alter cyprid behaviour. Cyprids from a single cohort were aged at two temperatures and then used in separate assays with light excluded (by wrapping the assay plate in aluminium foil) or not. Assays 'in light' were undertaken on a 16:8 L: D cycle and, additionally, they were placed on either a white or dark green tray. Day 0 cyprids were used immediately after removal from culture i.e. no holding temperature. Remaining cyprids were divided and aged to Day 1 separately at 6°C and 22°C (3.2.1). Separate 24-well plates for each assay condition were set up as follows:-

| Day 0 | Day 1 – aged at 6ºC | Day 1 – aged at 22ºC |
|---|---|---|
| Light excluded | Light excluded | Light excluded |
| In light* on light base | In light* on light base | In light* on light base |
| | In light* on dark base | In light* on dark base |

(* 'In light' means 16:8 L:D cycle.)

For each assay condition, 6 replicates of each of four water treatments - conspecific barnacle conditioned water (BCW), larval culture water, 3-isobutyl-1-methylxanthine (IBMX) $10^{-5}$ mol $l^{-1}$ and 0.45 $\mu$m filtered seawater - were tested. Cyprid behaviour was recorded at 24±2 hour intervals for 48 hours. The assays were repeated using a further cohort of cyprids; for the repeat, a further Day 0 assay was carried out in the light on a dark base.

**Experiment EM5 (Altered well surface)**

The experiment considered if the condition of the well surface would alter cyprid behaviour. Wells were altered either by roughening with fine sandpaper, or by adding a biofilm through immersion in a culture of *S. costatum* for 24 hours. Day 0 cyprids were used. Separate 24-well plate assays were set up for each assay condition using three cohorts of cyprids as follows:-

| Batch 1 | Batch 2 | Batch 3 |
|---|---|---|
| Unaltered wells | Unaltered wells | Unaltered wells |
| Roughened wells | Biofilmed wells | Roughened wells |
| | | Biofilmed wells |

Six replicates of four water treatments - conspecific BCW, IBMX $10^{-5}$ mol $l^{-1}$, IBMX $10^{-6}$ mol $l^{-1}$ and 0.45 $\mu$m filtered seawater - were tested on each plate. Cyprid behaviour was recorded after 24 hours.

**Experiment EM6 (Alternative assay method)**

The experiment considered if an alternative assay method would reduce floating and a series of mini assays were carried out as follows: 1) Four glass vials (Fisher, UK) each with 2 ml of 0.45 $\mu$m filtered seawater and 10 Day 2 cyprids; 2) Four 90 mm Petri dishes (Falcon, Fisher, UK) each with 9 ml of 0.45 $\mu$m filtered seawater and 20 Day 2

cyprids; 3) Four 35 mm Petri dishes (Falcon, Fisher, UK) each with 3 ml of 0.45 $\mu$m filtered seawater and 10 Day 4 cyprids in each; 4) Five 250 ml glass crystallizing dishes (Fisher, UK) each with 100 ml of 0.45 $\mu$m filtered seawater and 500 Day 0 cyprids, were prepared with one each of the following treatments: a) cleaned glass surface (soaked in 5% sodium hypochlorite solution for 60 minutes, rinsed thoroughly and heated to 100°C for 60 minutes); b) biofilmed glass surface (cleaned as above, then filled with several changes of seawater and left at room temperature for 3 days to develop a biofilm); c) conspecific SF treated glass surface (1 mg of extract brushed over the surface, air dried, then rinsed with filtered seawater 3 times); d) IBMX $10^{-5}$ mol $l^{-1}$ in seawater; e) unclean dish (previously used for cyprid storage, cleaned and rinsed in seawater). Cyprid behaviour was recorded after 24 hours.

**Experiment EM7 (Handling practices/IBMX induced settlement)**

The experiment considered if culture techniques and handling were contributing to floating behaviour in assays. Assays were set up simultaneously using *E. modestus* (cultured by the researcher) and *B. amphitrite* (cultured by an experienced technician). The experiment parameters were based on a previous experiment using *B. amphitrite* cyprids (Clare et al., 1995) describing the induction of settlement by IBMX in relation to cyprid age. Assays using Day 0, 3, 5, 7 and 9 cyprids from a single larval cohort were carried out with both species. *E. modestus* assays were carried out at 22°C and *B. amphitrite* assays were carried out at 28°C. IBMX solutions with a logarithmic concentration range from $10^{-8}$ mol $l^{-1}$ to $10^{-3}$ mol $l^{-1}$ were tested with 6 replicates of each treatment. Additionally, 6 replicates of 0.45 $\mu$m filtered seawater (as a negative control), and conspecific BCW (as a positive control) were prepared. Settlement and floating were recorded after 24 hours. The assays were repeated using a further larval cohort of each species.

## Effect of cyprid age on settlement

### Experiment EM8 (Selected ages)

The experiment was carried out using the 24-well plate assay (2.2.1) at 22ºC. One 24-well plate was set up for each cyprid age from a given cohort. Each plate had 8 replicates, each of 10 $\mu$g ml$^{-1}$ SF and BSA, and 0.45 $\mu$m filtered seawater. Three separate larval cohorts were used, with cyprid ages from each as follows: 1) Four ages of cyprid, Day 0, Day 1, Day 3 and Day 7; 2) Four ages of cyprid, Day 1, Day 3, Day 7 and Day 10; 3) Four ages of cyprid, Day 0, Day 1, Day 7 and Day 10. Settlement was recorded at 24±2 hour intervals for 48 hours.

For further assays, a 'Day 0 Hour 0 cyprid' was defined as the point when 70-80% of larvae had reached the cyprid stage in the culture.

### Experiment EM9 (Consecutive ages)

The experiment was carried out using the 24-well plate assay (2.2.1) at 22ºC. Eight consecutive ages of cyprid, Day 0 to Day 7, from a single cohort were tested. For each cyprid age, 8 replicates, each of 10 $\mu$g ml$^{-1}$ SF and 0.45 $\mu$m filtered seawater, were prepared. Settlement was recorded after 24 hours. The assays were repeated using a further cohort of cyprids.

## Trial nitrocellulose assays

### Experiment EM10

The experiment was carried out using the 24-spot nitrocellulose membrane assay (2.2.2) at 22ºC. Two assays, each with 400 Day 0 cyprids, were set up with a random selection of 12 spots treated with conspecific SF and the remaining spots having a negative control treatment of 50 mM Tris-HCl pH7.5 buffer. The concentration of SF was 50 $\mu$g ml$^{-1}$ in the first assay and 100 $\mu$g ml$^{-1}$ in the second (Matsumura et al., 2000). Settlement was recorded after 24 hours.

## 4.2.2 *Semibalanus balanoides*

### Cyprid origin

As the laboratory culture of *S. balanoides* cyprids was unsuccessful, wild cyprids were used. They were collected on May 3rd, May 15th and May 24th (3.2.2), although insufficient larvae were collected on May 24th for assays.

### Experimental design

### Experiment SB1 (Temperature and concentration of SF)

The assays were carried out with cyprids collected on May 3rd 2002, and repeated with those collected on May 15th 2002. The assays were set up the day after the collection of cyprids. The method used was the 24-well plate assay (2.2.1) and four 24-well plates were set up for each of five different temperatures, 5, 11, 15, 19 and 22°C. For each temperature, 8 replicates, each of 4 concentrations, 10, 20, 30 and 40 $\mu$g ml$^{-1}$ of conspecific SF and BSA, were prepared. Additionally, 8 replicates of 0.45 $\mu$m filtered seawater were prepared for each temperature/concentration combination. Settlement was recorded at 24±2 hours for a period of 21 days.

### Experiment SB2 (Nitrocellulose membrane trial assays)

The experiments were carried out using cyprids collected on May 15th 2002. The cyprids were aged for 5 days at 6°C before using in assays. The experiment was carried out using the 24-spot nitrocellulose membrane assay (2.2.2) at 19°C. Two assays, each with 400 cyprids, were set up each with a random selection of 12 spots treated with conspecific SF with the remaining spots having a negative control treatment of 50 mM Tris-HCl pH7.5 buffer. The concentration of SF was 50 $\mu$g ml$^{-1}$ in the first assay, and 100 $\mu$g ml$^{-1}$ in the second. Settlement was scored at 48±2 hour intervals for 8 days.

## 4.3 Results

### 4.3.1 *Elminius modestus* experiments

#### Optimum temperature and concentration of conspecific settlement factor

**Experiment EM1 (Selected temperatures between 5 and 27°C)**

Settlement did not occur at less than 19°C and was highest at 22°C for both ages of cyprids. Overall, greatest settlement occurred in the first 24-hour period and trends continued in cumulative settlement at the 48- and 72-hour observations. Figure 4.1 illustrates the results of Cohort 1 at the 24-hour observation for Day 0 cyprids. Where settlement occurred, wells that had been treated with SF generally had higher settlement than those treated with BSA or the seawater control; the exceptions were Day 1 cyprids with SF and BSA concentrations of 30 and 40 $\mu$g ml$^{-1}$. Settlement of Day 0 cyprids at 22°C in SF-treated wells was highest at a concentration of 10 $\mu$g ml$^{-1}$, with more than 50%, 70% and 80% settlement at 24, 48 and 72 hours respectively. Settlement of Day 1 cyprids was lower than the comparative Day 0 settlement at all temperatures and treatments. Statistical analysis was unnecessary for assays carried out ≤15°C, as the results were clearly not significant. Kruskal-Wallis statistics carried out on all remaining data indicated that settlement by Day 0 cyprids was significant at 22°C except for Cohort 1 at a SF concentration of 20 $\mu$g ml$^{-1}$ and the 72-hour observation of Cohort 2. Settlement by Day 1 cyprids was generally not significant.

**Experiment EM2 (Effect of SF concentration between 0-150 $\mu$g ml$^{-1}$)**

For both cohorts maximum settlement occurred in 10 $\mu$g ml$^{-1}$ SF. Overall settlement was higher for Cohort 1 than Cohort 2. Figure 4.2 shows the results of the assay using Cohort 1 larvae. Kruskal-Wallis statistics indicated that settlement was significantly different at different concentrations of SF ($P = 0.006$, Cohort 1; and 0.024, Cohort 2).

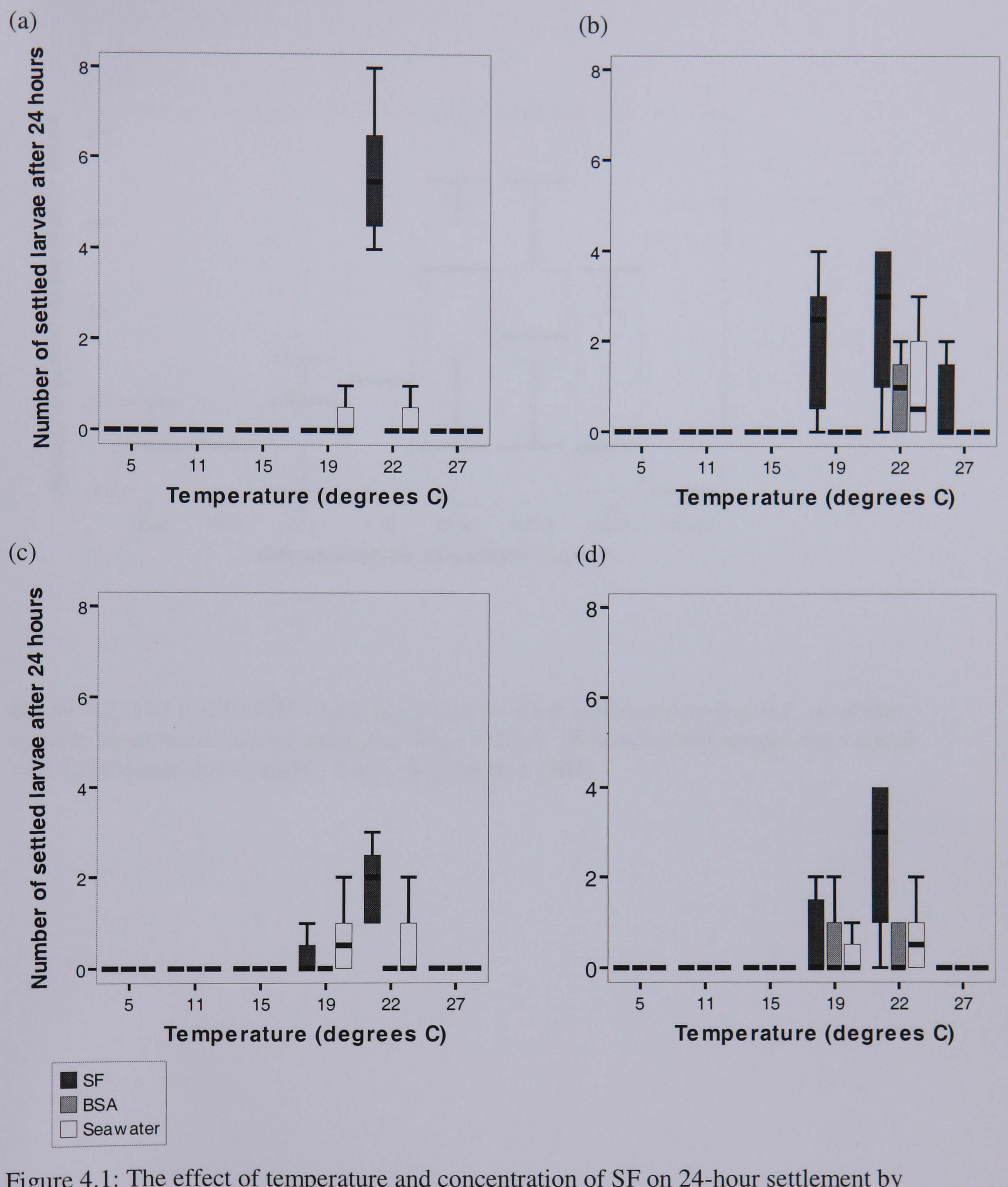



Figure 4.1: The effect of temperature and concentration of SF on 24-hour settlement by Day 0 *E. modestus* cyprids compared to BSA and seawater controls. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 8. Conspecific SF and BSA concentrations are (a) 10 $\mu$g ml$^{-1}$, (b) 20 $\mu$g ml$^{-1}$, (c) 30 $\mu$g ml$^{-1}$, (d) 40 $\mu$g ml$^{-1}$. (Experiment EM1)

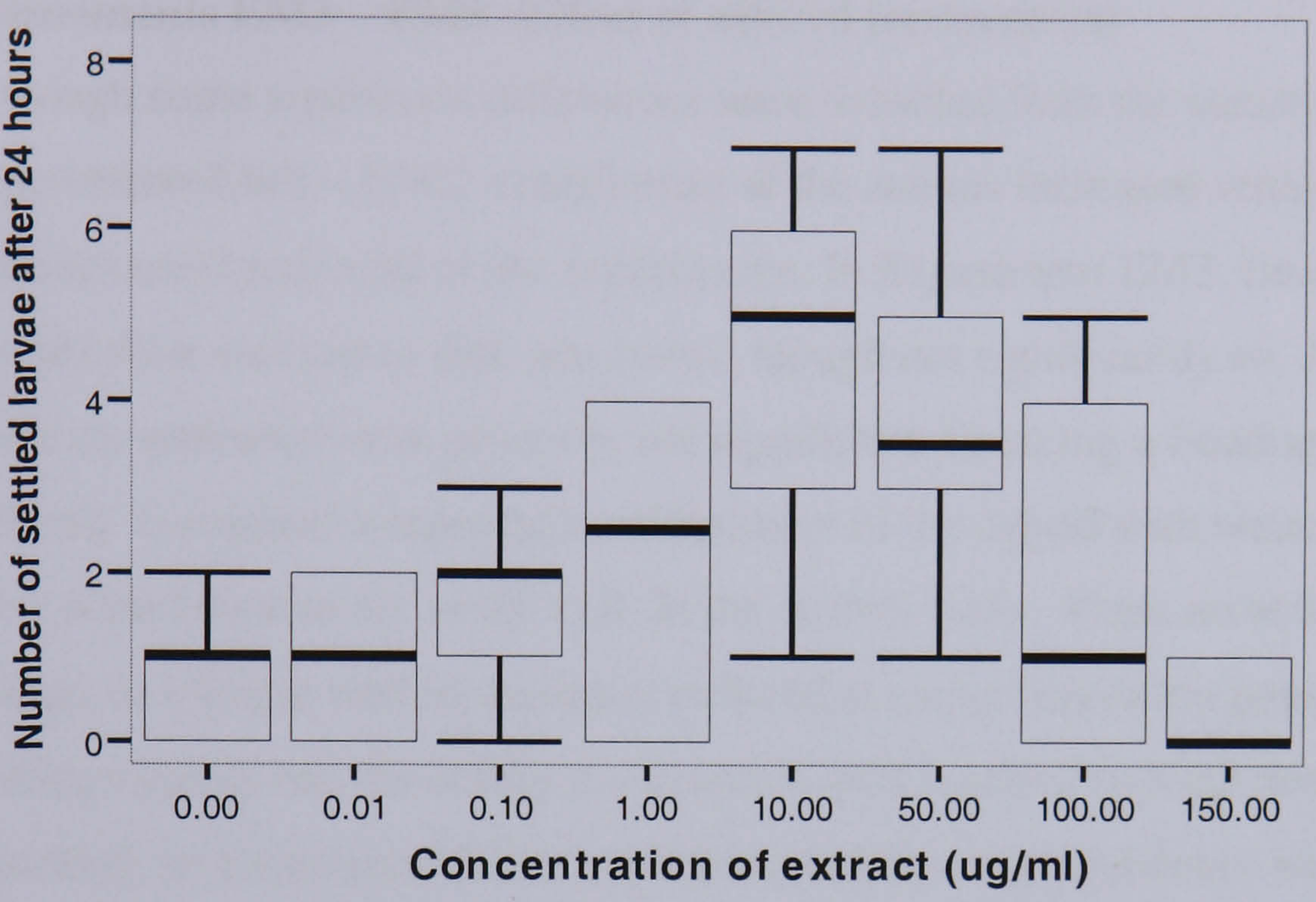



Figure 4.2: The effect of SF concentration on 24-hour settlement by Day 0 *E. modestus* cyprids. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 6. (Experiment EM2)

## Attempts to reduce the incidence of cyprid floating

### Experiments EM3 – EM5 (Effect of altered parameters)

Although some significant differences were recorded from the statistical analyses of Experiments EM3 – EM5, overall none of the actions increased settlement levels and floating continued in all of the experiments. In Experiment EM3, floating in wells where cyprids were coerced to sink was lower, though not significantly so. Additionally, the effect on settlement was generally not significant. Coercing a floating cyprid to sink was difficult. It required a repeated bombardment of the cyprid with water droplets, causing great disturbance to the assay well. In the control wells, where no action was taken, floaters in a single well increased or reduced at each observation point. Thus, the floating cyprids had the ability to overcome their inactive floating state and resume searching. In Experiment EM4, settlement and floating behaviours were the same for Day 1 cyprids aged at 22°C and 6°C. Different light regimes did not significantly alter cyprid behaviour at Day 0 or Day 1. In Experiment EM5, fine perspex shavings remained in wells that had been roughened with sandpaper and this was an undesirable influence on cyprid behaviour. Biofilmed wells were stagnant. The actions were not successful at reducing cyprid floating and the procedures were not adopted.

### Experiment EM6 (Mini assays using alternative methods)

Settlement did not occur in the glass vials and between 40-70% of cyprids floated. Settlement did not occur in the 90 mm Petri dishes, and was ca. 5% in 35 mm Petri dishes. Floating in these assays was difficult to determine, as the Petri dish lid was in contact with the upper water surface. Floating on removal of the lid was 30-60% in both sets of assays. Floating behaviour was observed in the larger crystallising dish assays, although it was lower than the smaller assays. Settlement was similar to 24-well plate assays of comparable treatments, although metamorphosed individuals had poor surface adhesion and some had partially detached. These alternative methods were not adopted.

**Experiment EM7 (Handling practices/IBMX induced settlement)**

The experiment was undertaken primarily to consider if culture handling practices influenced cyprid behaviour in assays. The effects of IBMX are reported for both species, and results compared to consider if handling affected assay results. The results for both *B. amphitrite* cohorts were comparable, though they were not for *E. modestus*, with Cohort 2 settlement almost nil throughout.

***B. amphitrite***

IBMX at a concentration of $10^{-5}$ mol $l^{-1}$ induced greatest 24-hour settlement at the majority of ages. The exception was Day 7 cyprids where greatest settlement was induced by IBMX $10^{-8}$ mol $l^{-1}$. Overall the level of 24-hour settlement induced by IBMX increased with increasing cyprid age. The 48-hour settlement peaked at either IBMX $10^{-6}$ mol $l^{-1}$ (Day 0, 7 and 9) or IBMX $10^{-5}$ mol $l^{-1}$ (Day 3 and 5). For all cyprid ages, the level of floating at the 24-hour observation was highest at IBMX $10^{-3}$ mol $l^{-1}$, decreasing with reducing IBMX concentrations. Floating was low in control treatments. Kruskal-Wallis statistics comparing different IBMX concentrations for each cyprid age separately indicated that settlement and floating were significantly different at all ages (Table 4.1).

***E. modestus***

Cohort 1 24-hour settlement was low (< 20 - 30%) for all cyprid ages and all water treatments. Settlement for Day 0 and Day 3 cyprids was nil after 24 hours. Day 5 24-hour settlement was low at IBMX $10^{-5}$ mol $l^{-1}$ and marginally higher settlement at $10^{-6}$ mol $l^{-1}$, while for Day 7 cyprids the higher settlement occurred at IBMX $10^{-7}$ mol $l^{-1}$ and for Day 9 at IBMX $10^{-5}$ mol $l^{-1}$. Floating was high at both observations points for all ages of cyprid and all IBMX concentrations, and in control wells. Kruskal-Wallis statistics comparing different IBMX concentrations for each cyprid age separately lacked a clear interpretation (Table 4.1).

| Species | Age | Kruskal-Wallis tests P values | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Settlement | | | | Floating | | | |
| | | Cohort 1 | | Cohort 2 | | Cohort 1 | | Cohort 2 | |
| | | 24hour | 48hour | 24hour | 48hour | 24hour | 48hour | 24hour | 48hour |
| *B.amphitrite* | Day 0 | <0.001 | 0.001 | 0.027 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | Day 3 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.001 | <0.001 |
| | Day 5 | 0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | Day 7 | 0.001 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | Day 9 | 0.005 | 0.001 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | 0.005 |
| *E.modestus* | Day 0 | 1.000 | 0.525 | 0.004 | 0.034 | 0.001 | 0.015 | 0.010 | 0.003 |
| | Day 3 | 1.000 | 0.123 | 0.085 | 0.001 | 0.101 | 0.139 | 0.062 | 0.069 |
| | Day 5 | 0.072 | 0.051 | 0.002 | 0.002 | 0.001 | 0.002 | 0.040 | 0.220 |
| | Day 7 | 0.199 | 0.574 | 0.530 | 0.014 | 0.010 | 0.021 | 0.005 | 0.068 |
| | Day 9 | 0.188 | 0.067 | 0.007 | 0.021 | 0.019 | 0.003 | 0.086 | 0.165 |

Table 4.1: Summary results (P values) of Kruskal-Wallis tests comparing settlement in response to different IBMX concentrations and water treatments for each cyprid age separately (Experiment EM7).

## Effect of cyprid age on settlement

### Experiments EM8 (Selected ages)-EM9 (Consecutive ages)

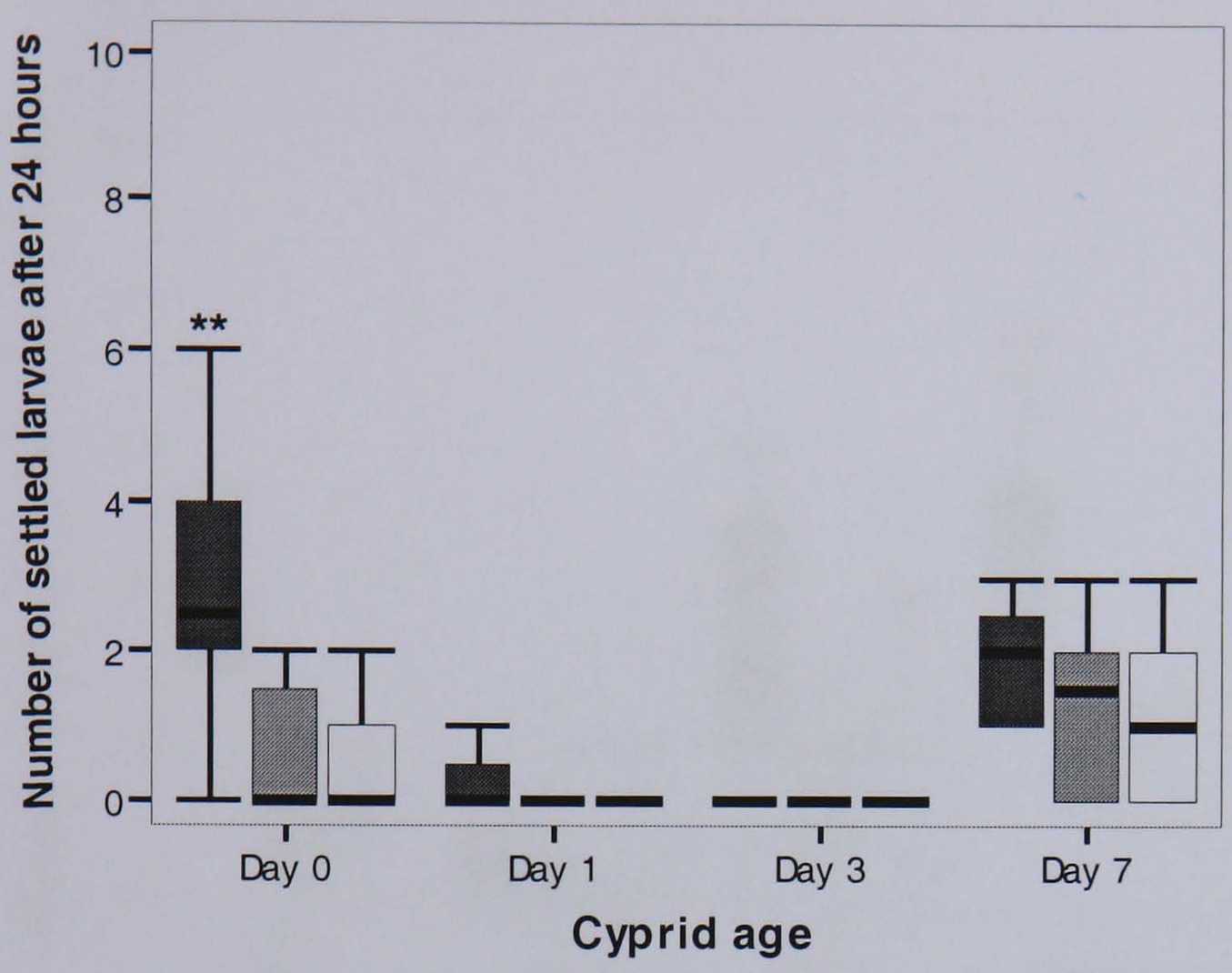
Figure 4.3 illustrates the results of Experiments EM8 and EM9. Settlement by *E. modestus* cyprids of increasing age resulted in a distinct pattern, particularly when induced by the presence of conspecific SF. A trend of high settlement for Day 0, reducing for Day 1, low settlement for Day 2-4, and rising again for older cyprids was observed. The trend was most easily observed in Experiment EM9, where consecutive ages from Day 0 to Day 7 were tested. Settlement after 24 hours was always higher in SF-treated wells than in the seawater controls, but the difference between treated and control wells decreased with increasing cyprid age. For any age, inter-assay variability in settlement was up to 40%. Kruskal-Wallis statistics comparing settlement between SF treated and controls for each cyprid age indicated that the results were significant for all assays carried out using Day 0 cyprids (P = 0.007 EM8 Cohort 1, 0.006, EM8 Cohort 3; 0.004, EM9 Cohort 1; 0.001, EM9 Cohort 2) and some other ages in certain assays.

## Nitrocellulose membrane experiments

### Experiment EM10 (50 and 100 $\mu$g spot$^{-1}$ SF concentration at 22°C)

Figure 4.4 illustrates the results of Experiment EM10. Settlement after 24 hours was higher on SF-treated spots than the control spots for both SF concentrations tested. Settlement on SF-treated spots was higher in the 50 $\mu$g spot$^{-1}$ assay than the 100 $\mu$g spot$^{-1}$. Control settlement in both assays was similar. Mann-Whitney U statistics comparing SF-treated spots to the control indicated that settlement was significantly higher on the SF-treated spots compared to the control at 50 $\mu$g spot$^{-1}$ ($P < 0.001$), but not at 100 $\mu$g spot$^{-1}$.

(a)




(b)

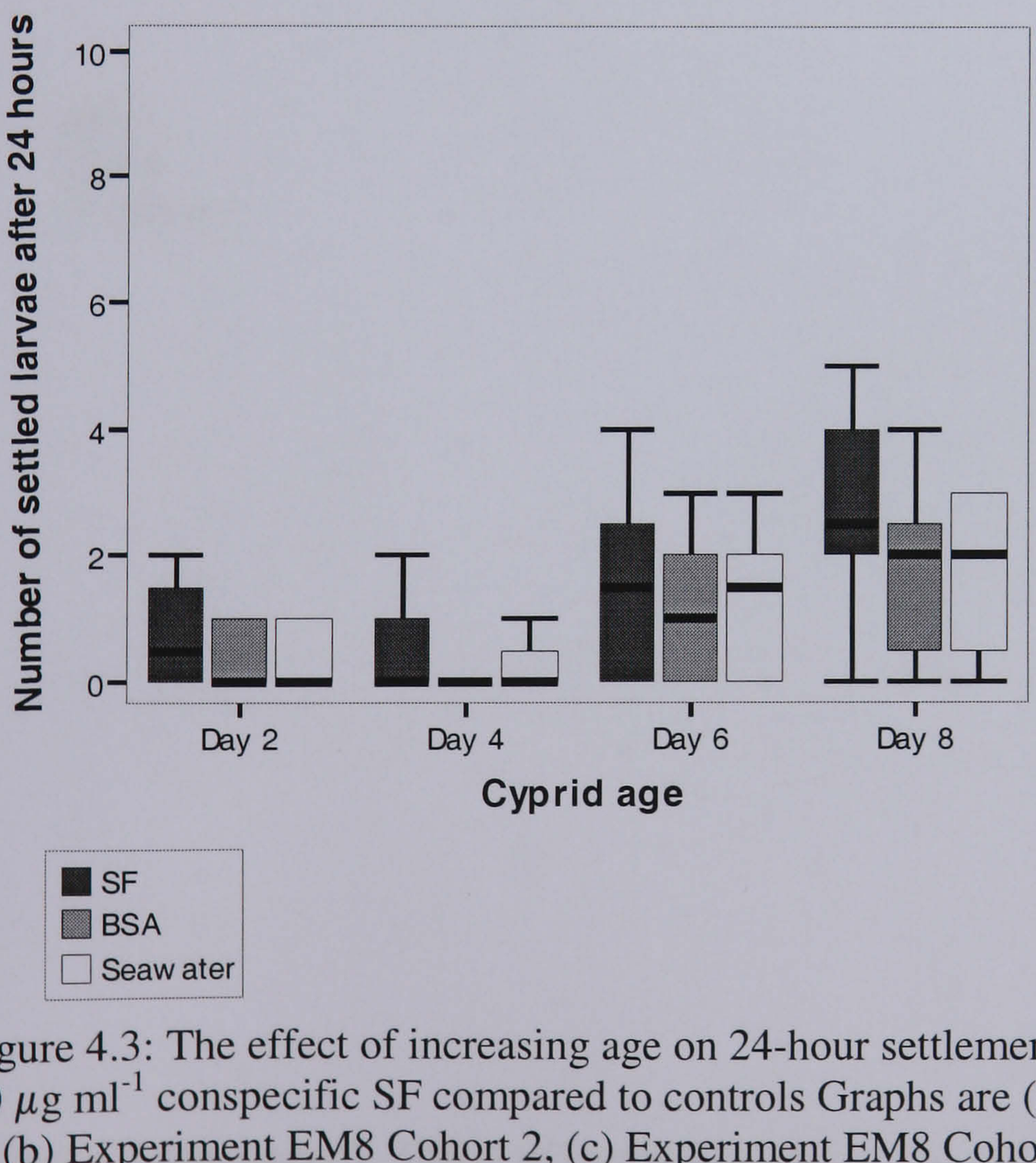



Figure 4.3: The effect of increasing age on 24-hour settlement by *E. modestus* cyprids to 10 $\mu$g ml$^{-1}$ conspecific SF compared to controls Graphs are (a) Experiment EM8 Cohort 1, (b) Experiment EM8 Cohort 2, (c) Experiment EM8 Cohort 3, (d) Experiment EM9 Cohort 4 and (e) Experiment EM9 Cohort 5. Controls are 0.45 $\mu$m filtered seawater in all and 10 $\mu$g ml$^{-1}$ BSA in (a), (b) and (c). Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 6. **P<0.01; ***P<0.005. (Experiment EM8 and EM9)

(c)

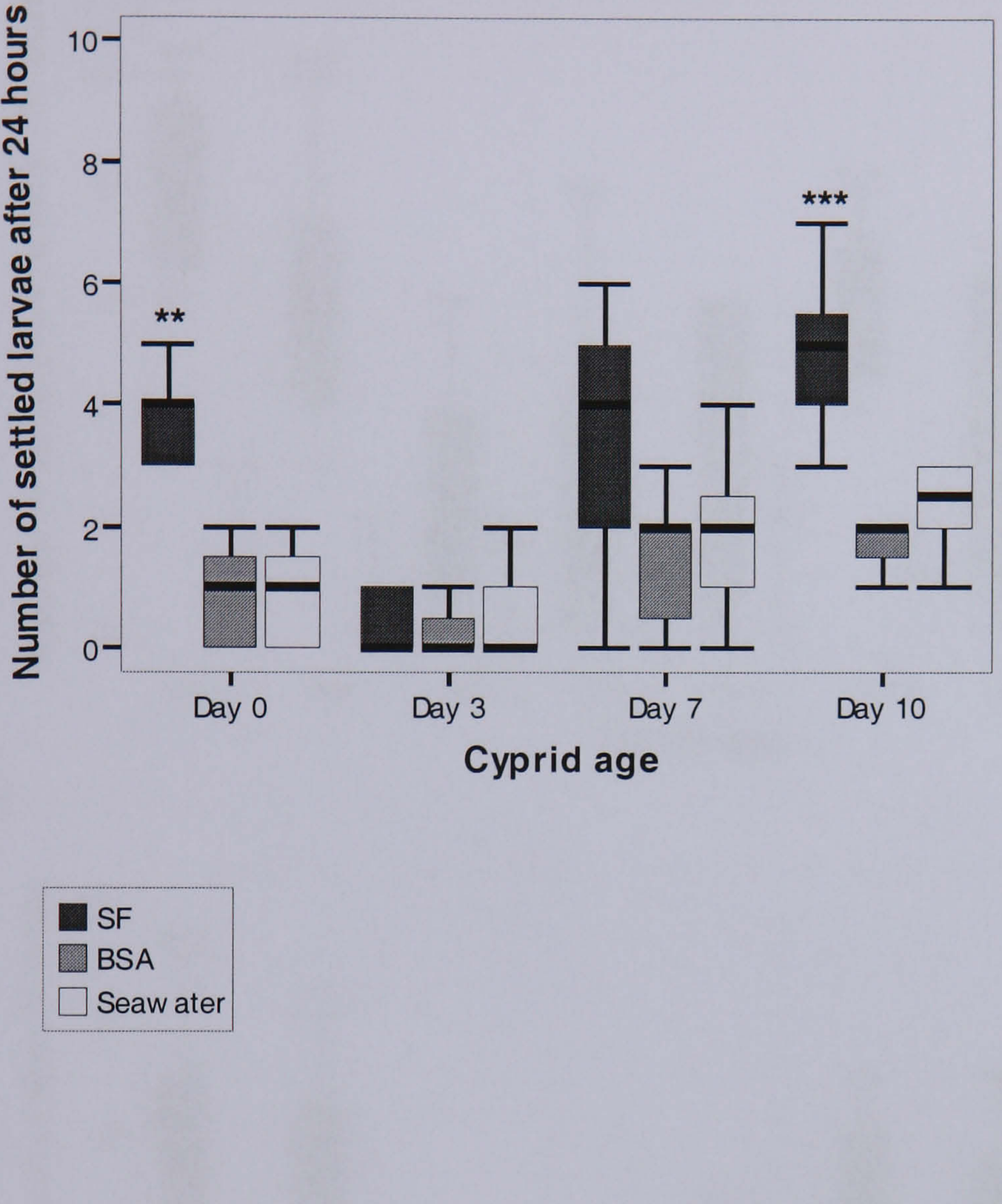
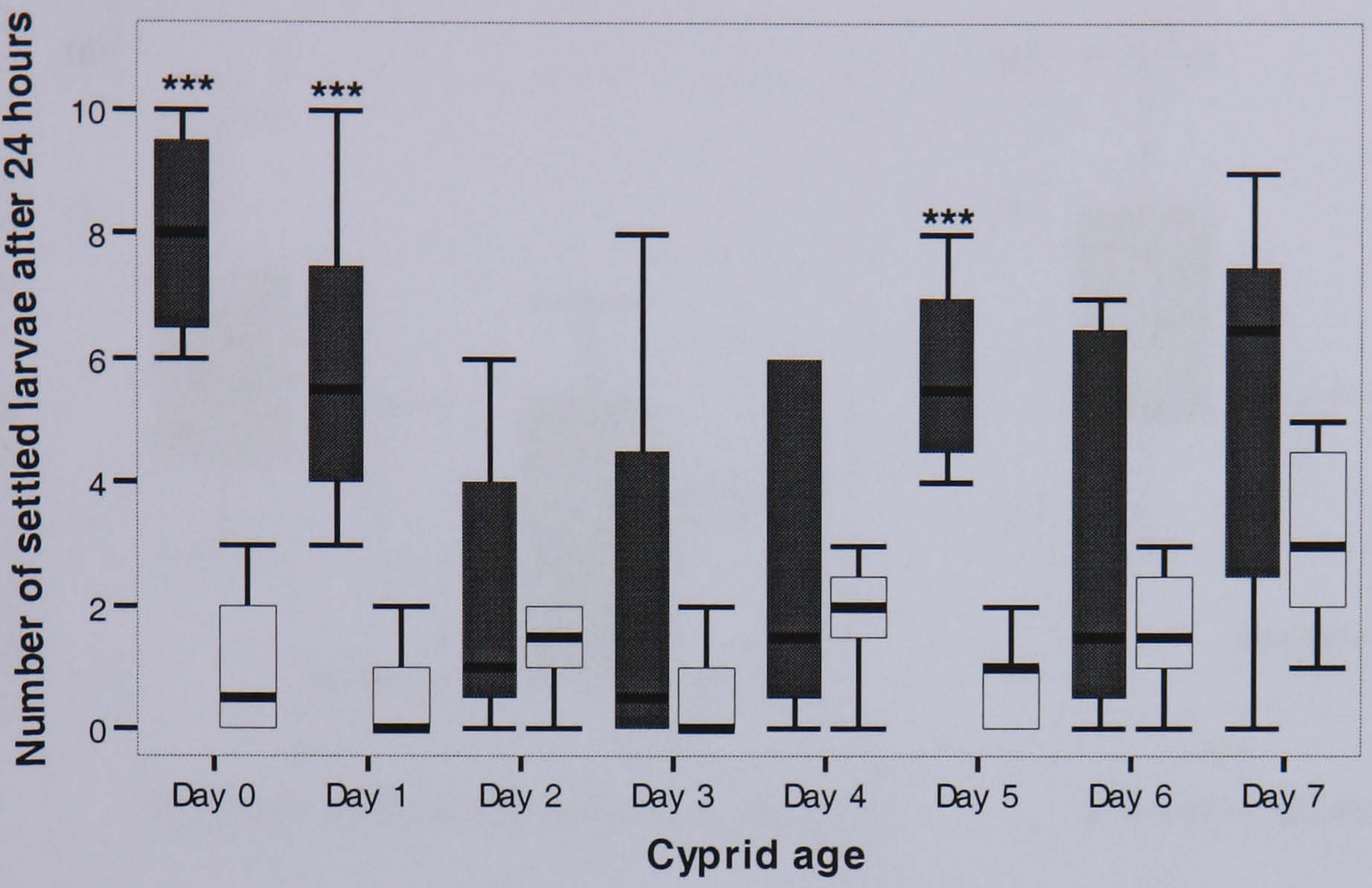



Figure 4.3 (continued): Effect of increasing age on 24-hour settlement of *E. modestus* cyprids to 10 $\mu$g ml$^{-1}$ conspecific SF. The graphs is (c) Experiment EM8 Cohort 3.

(d)




(e)

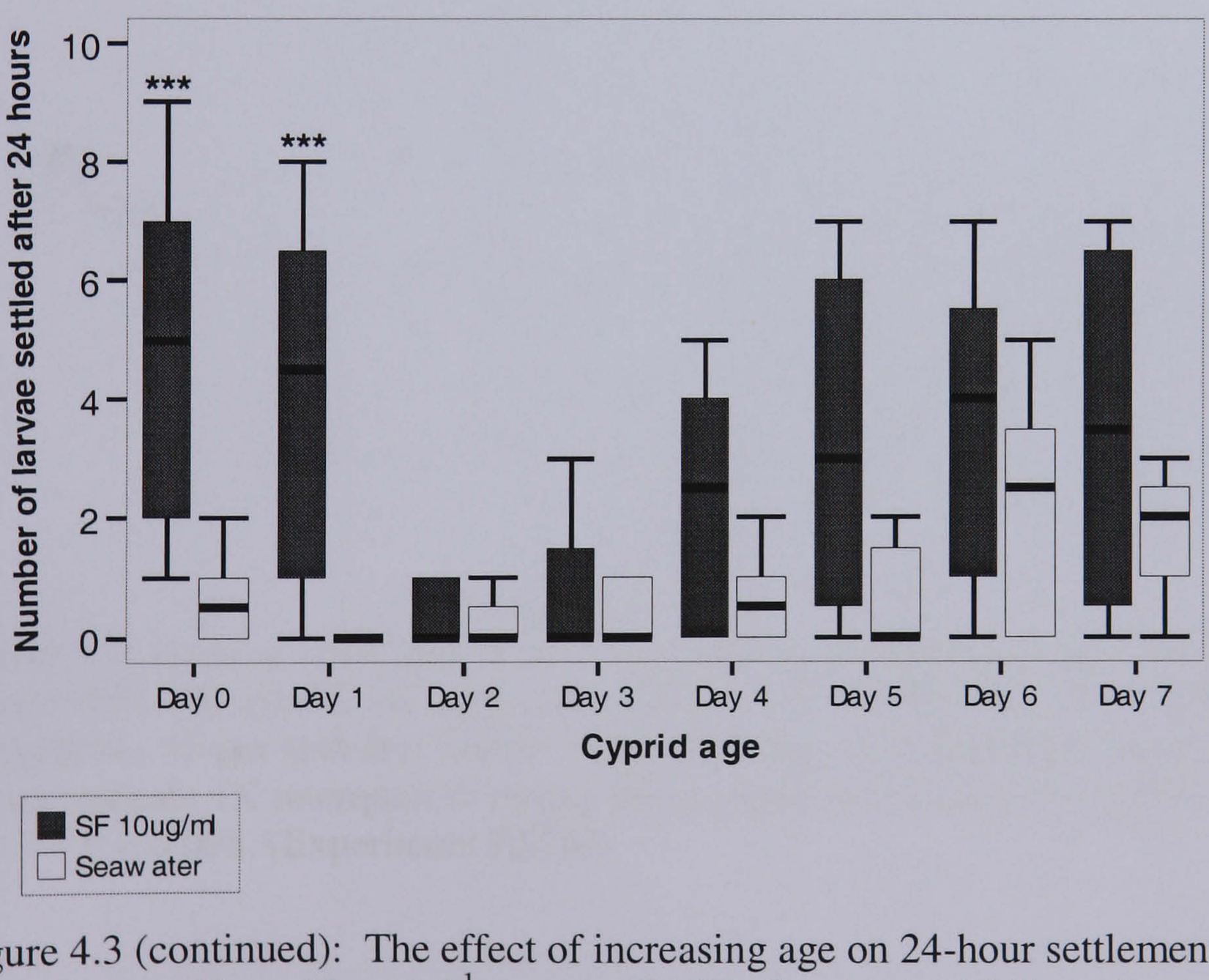



Figure 4.3 (continued): The effect of increasing age on 24-hour settlement of *E. modestus* cyprids to 10 $\mu$g ml$^{-1}$ conspecific SF. Graphs are (d) Experiment EM9 Cohort 4 and (e) Experiment EM9 Cohort 5.

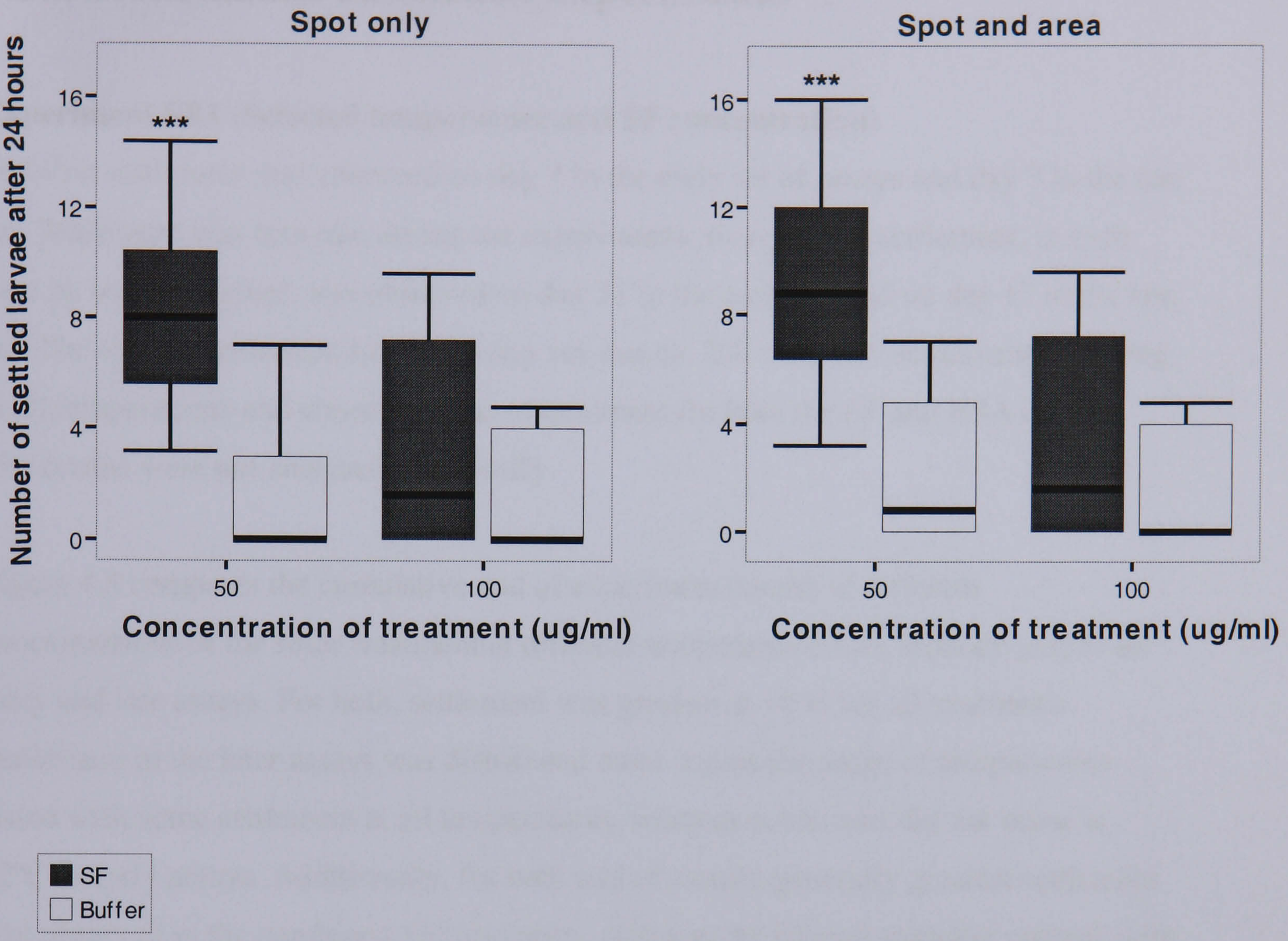



Figure 4.4: 24-hour settlement of Day 0 *E. modestus* cyprids in nitrocellulose membrane assays with 400 cyprids per assay carried out at 22ºC with either 50 or 100 $\mu$g of conspecific SF per spot and 'buffer only' control spots. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 12. ***P < 0.005. (Experiment EM10)

## 4.3.2 *Semibalanus balanoides* experiments

**Experiment SB1 (Selected temperature and SF concentration)**

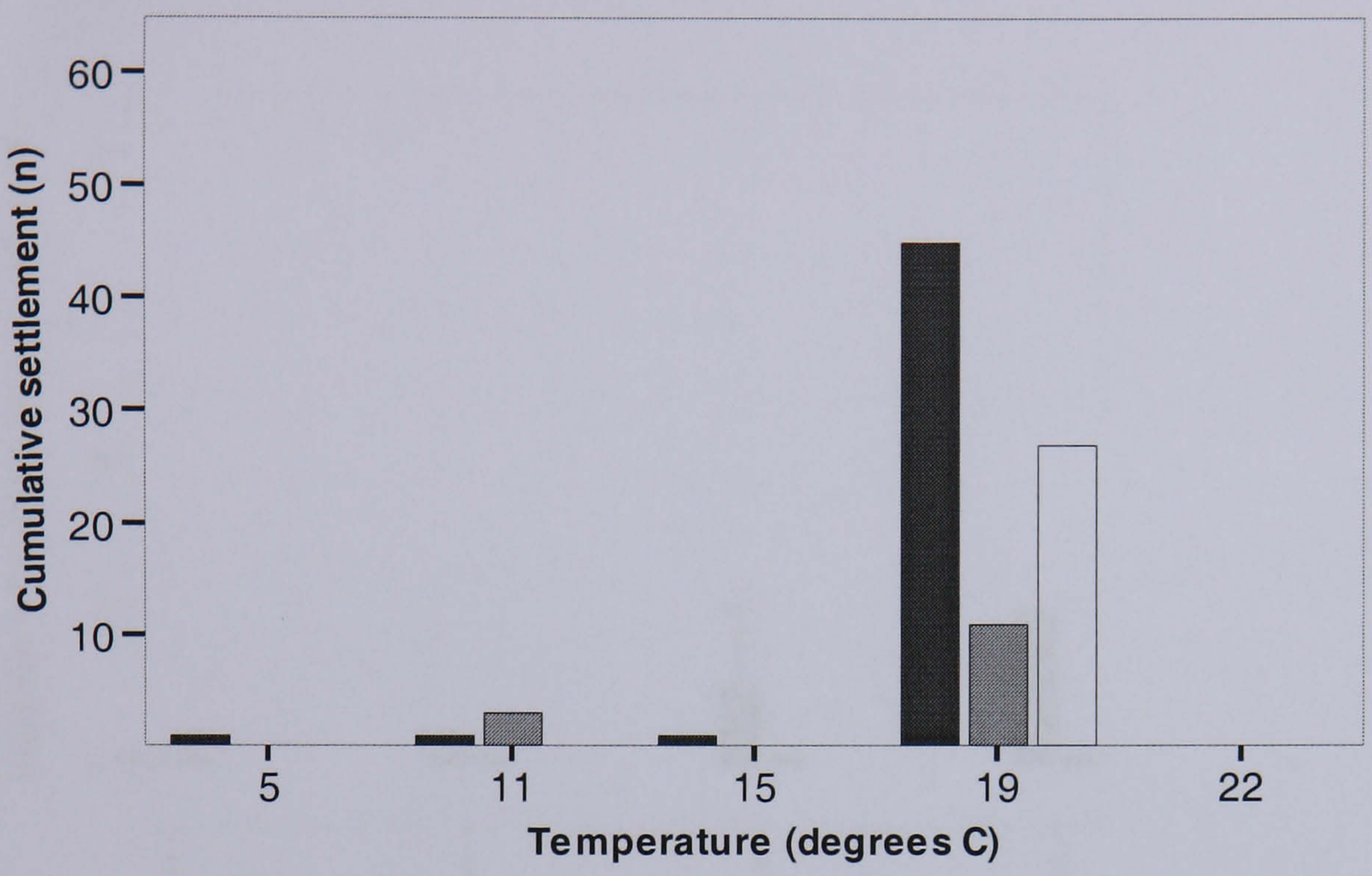
The first settlement was observed on day 7 in the early set of assays and day 3 in the late set. Settlement was sporadic during the experiments, though final settlement, in each case by one individual, was observed on day 21 in the early set and on day 17 in the late set. The overall settlement for each assay set was ca. 2%, with low settlement occurring at all temperatures and concentrations of treatment for both the SF and BSA control. The results were not analysed statistically.

Figure 4.5 compares the cumulative end of experiment results of different concentrations of the same treatment at different temperatures with separate graphs for early and late assays. For both, settlement was greatest at 19°C for all treatments. Settlement in the later assays was distributed more across the range of temperatures tested with some settlement at all temperatures, whereas settlement did not occur at 22°C in early assays. Additionally, for both sets of assays, generally greatest settlement was observed in the combined SF treatments, followed by filtered seawater control, with lowest settlement in the combined BSA treatments. The exceptions were at 11°C in early assays, where more cyprids settled in the combined BSA controls, and 15°C in late assays, where more settled in the combined seawater controls.

**Experiment SB2 (Nitrocellulose trial assays)**

Figure 4.6 illustrates the results of the nitrocellulose membrane assays. First settlement was observed at day 6 in the 50 $\mu$g spot$^{-1}$ assay and day 4 in the 100 $\mu$g spot$^{-1}$ assay. Once settlement had occurred, settlement was higher on the treated spots than on the control for both assays at all observations, although Mann Whitney U statistics indicated that the difference was not significant.

(a)




(b)

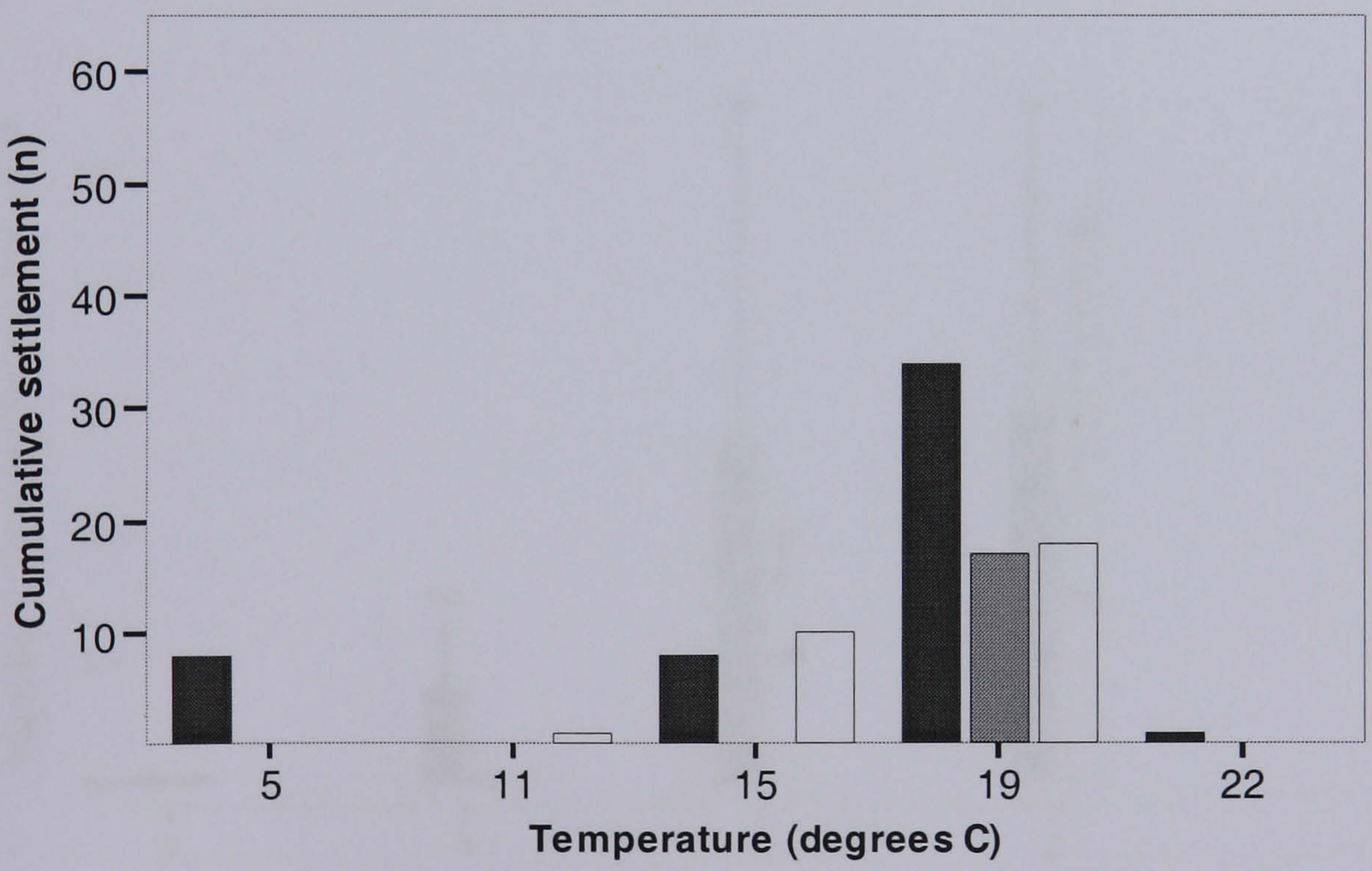
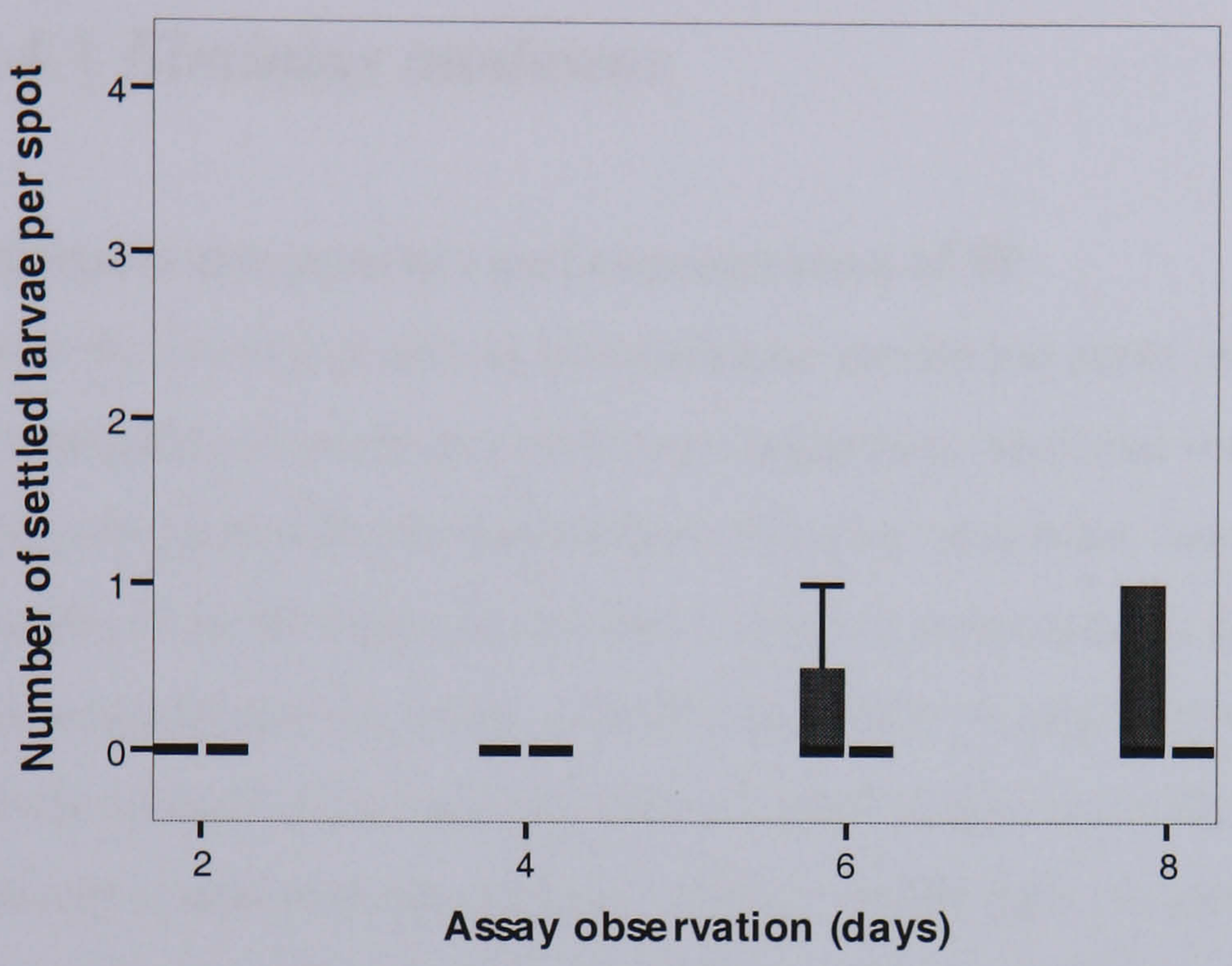






Figure 4.5: Cumulative end of experiment settlement of wild *S. balanoides* cyprids in response to conspecific SF, and seawater and BSA controls, in various 24-well assays carried out at different temperatures. Cyprids were collected (a) May3rd 2002 and (b) May 15$^{th}$ 2002. (Experiments SB1)

(a)




(b)

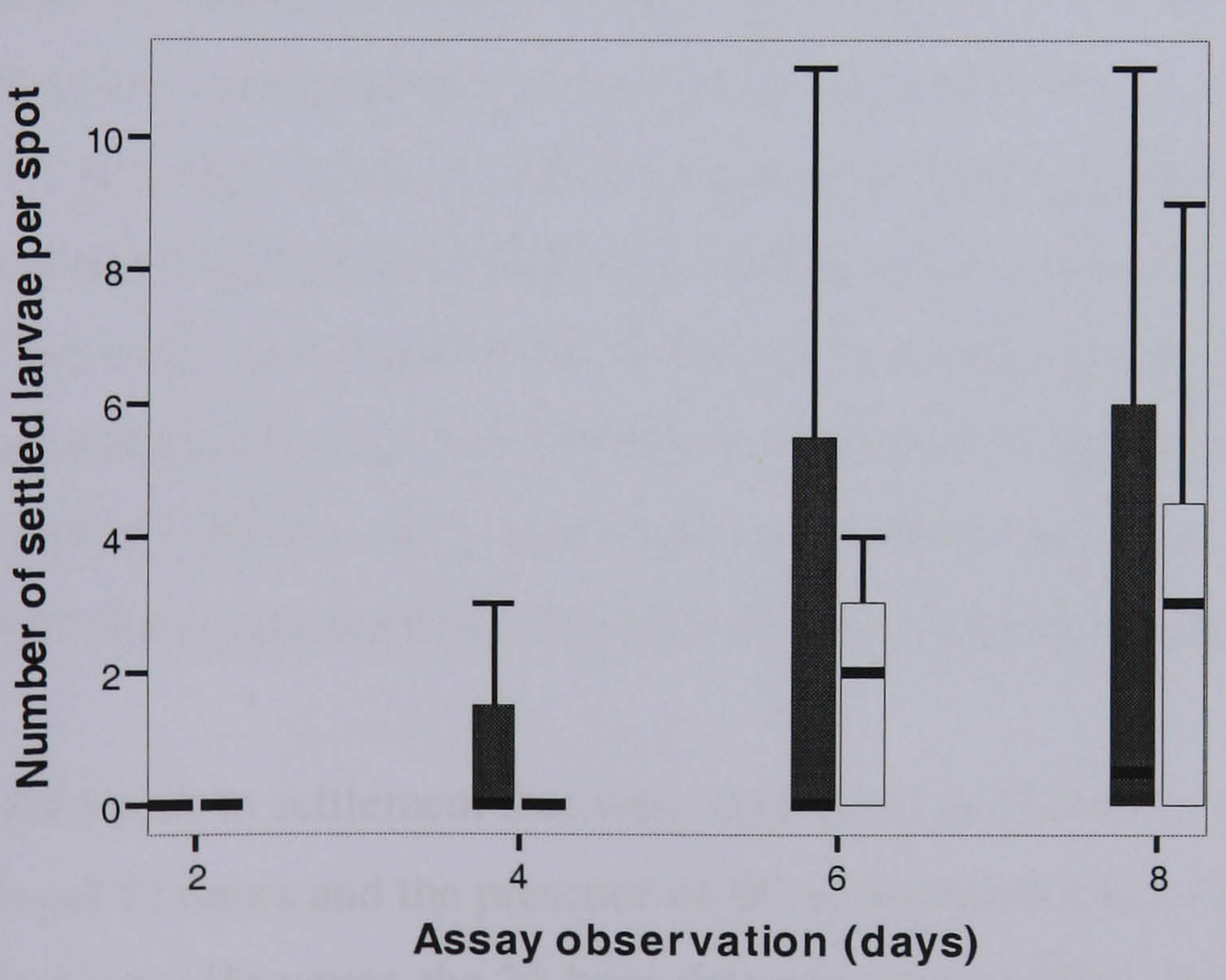



Figure 4.6: *S. balanoides* settlement on SF treated and untreated areas of nitrocellulose membrane. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 12. SF concentrations are (a) 50 $\mu$g spot$^{-1}$ and (b) 100 $\mu$g spot$^{-1}$. (Experiment SB2)

# 4.4 Discussion

## 4.4.1 *Elminius modestus*

### Optimum temperature and concentration of SF

Both the 24-well plate and nitrocellulose membrane assay methods were suitable for investigating *E. modestus* settlement behaviour. Maximal settlement occurred at 22°C and coincided with the temperature of the larval culture. Larval development and settlement in the field occurs over a range of temperatures. Although UK summer mean sea temperatures are likely to be less than 22°C (Cooper, 1958; Muxagata et al., 2004), newly settled larvae may experience higher temperatures during periods of immersion as a result of seasonal and climate variation. While peak settlement occurred at 22°C, temperatures were selected at 3 – 5°C intervals, such that the true peak may be marginally different. However, an underlying purpose of the experiments was to determine an appropriate temperature for all further assays and this has been achieved to ±5°C accuracy. It was convenient to carry out culture and assays in the one available incubator simultaneously, such that further experiments that could improve the accuracy of the result were superfluous. While results at temperatures other than 22°C were contradictory at times, the 22°C assays most reliably indicated significant differences in settlement between SF treated wells and controls, and was a compounding reason to adopt this temperature for both 24-well plate and nitrocellulose membrane assays.

Differences in settlement that were significant at 24 hours were generally significant at 48 and 72 hours and the presence of SF would seem a key factor required to induce settlement. However, the 24-hour data were most useful. With increasing assay duration, settlement may have been induced by larva-larva interactions (Matsumura et al., 1998c; Head et al., 2003), the presence of cyprid footprints from searching activity (Yule and Walker, 1985; Clare et al. 1994), and the development of a biofilm (e.g. Wieczorek et al. 1995; Harder et al., 2001b). Optimal settlement occurred at a SF concentration of 10 $\mu$g

$ml^{-1}$ (EM2), a result that was comparable to *B. amphitrite* research (Matsumura et al., 2000).

**Investigating if floating could be reduced**

Floating by *E. modestus* cyprids occurred in all 24-well plate assays throughout the research irrespective of well treatment and at times accounted for 100% of behaviour in individual wells. The concern was that floating (possibly as a result of air trapped beneath the carapace, or entrapment in the meniscus as a result of vigorous swimming in circular sweeps by active cyprids) suspended individuals from searching, and thus from settlement, which could bias results. The pipetting of water droplets onto larvae, a method that was successful with floating *Adalaria proxima* larvae (Gastropoda: Nudibranchia) (Todd et al., 1991), was ineffective, as larvae had to be repeatedly bombarded causing great disturbance to wells, which itself may have influenced behaviour. Other measures were also ineffective.

While floating may be an unavoidable effect of laboratory rearing and handling, it could be a naturally-occurring behaviour. Some floating cyprids (< 200 - 300) were observed in the culture medium when cyprids had not been handled, though more floated after removal and during storage (20-50%). Knight-Jones (1953a) commented that wild *E. modestus* cyprids were easy to separate from the trawled plankton samples, because they became trapped in the meniscus when left undisturbed in dishes. The cause is not considered by the author, but trawling and handling of samples may have contributed to the behaviour. Harder et al. (2001a) recorded floating behaviour by *B. amphitrite* cyprids in assays testing the effects of biofilm. He observed that under control conditions (i.e. without a biofilm), while cyprids floated briefly, they resumed searching easily. He hypothesised that, in the presence of a biofilm, cyprids were entrapped by a thin organic layer formed on the water surface, as a result of bacterial decomposition of particulate organic matter in the biofilm matrix. The presence of SF, a protein mix with associated sugars, would encourage the development of a biofilm, and thus from Harder's hypothesis floating would be higher in SF treated wells than blank controls. However, the reverse was observed. Repeated observation of assays showed that the

level of floating may increase or decrease in any single well during the assay. *E. modestus* cyprids, like control *B. amphitrite* observed by Harder, had the ability to overcome their floating state. Cyprids, detecting the presence of a suitable settlement cue, i.e. SF, maintained searching, suggesting that the inactive state of floating in the absence of a cue may be an active choice. As cyprids were able to resume searching, and high settlement did occur when conditions were favourable, floating behaviour did not detract from the value of the assay result and statistical analysis was valid. Floating cyprids, unlike dead cyprids, were not excluded when calculating percentage settlement per well.

**IBMX-induced settlement**

The presence of IBMX, a phosphodiesterease inhibitor, that raises intracellular levels of cyclic nucleotides, including cyclic AMP, may induce settlement. It has been proposed that cyclic AMP is involved in the signal transduction pathway resulting in settlement (Clare et al., 1995). The virtually nil settlement by *E. modestus* Cohort 2 may have been due to larval quality. The low settlement by Cohort 1 may indicate that IBMX was not an effective inducer of settlement, or that the concentrations tested were inappropriate for this species. Differences in floating behaviour by both species provided further insight. Substantial *B. amphitrite* floating occurred at all ages in the higher concentrations of IBMX, but declined with reducing concentrations. As higher concentrations of IBMX are considered to be inhibitory to settlement (Clare et al., 1995), the floating behaviour may be associated with the toxic environment. *E. modestus* floating was generally high for all ages and concentrations of IBMX, suggesting a greater sensitivity to the compound. Thus, a reduced concentration of IBMX may induce settlement. However, further experiments were not undertaken, as the results of the experiment met the objective to clarify if handling practices were causative of cyprid floating and the *B. amphitrite* results suggested that they were not.

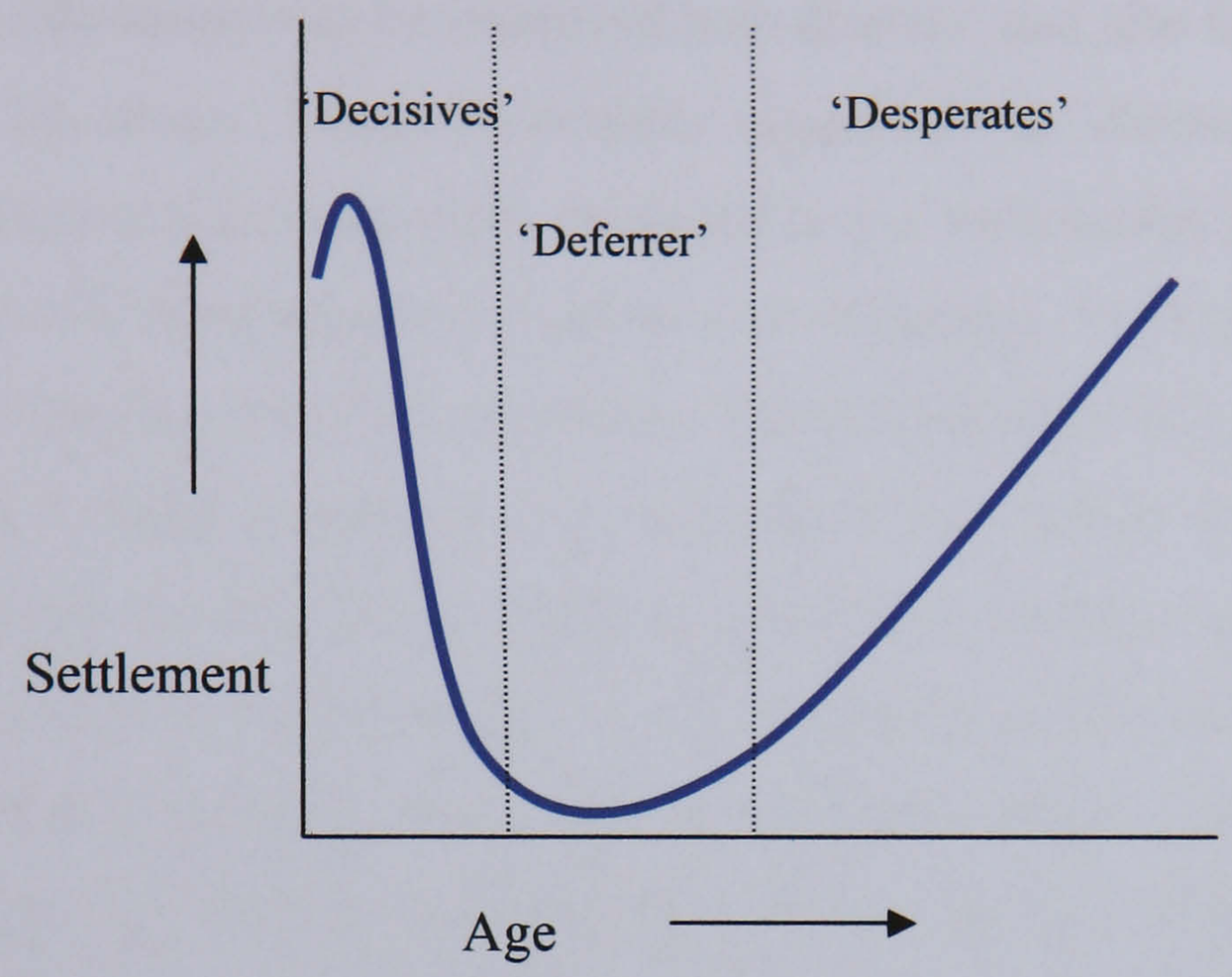
**The effects of cyprid age on settlement**

*E. modestus* cyprids settled at a high rate when young and relatively old, while at intermediate ages (Day 2-3) settlement was low. This trend has not previously been

reported for marine invertebrate larvae. In contrast, the 'desperate larva' hypothesis, so named by Toonen and Pawlik (1994), was proposed by Wilson (1953) and Knight-Jones (1953b), after observation of the development of non-feeding pelagic larvae that exhaust energy reserves as they develop in the plankton. *B. amphitrite* larvae settle at a low rate when young and settlement increases with age with loss of discriminatory ability (Rittschof et al., 1984; C. Hellio pers.comm.), which is consistent with the 'desperate larvae' hypothesis. Satuito et al. (1996) also found that *B. amphitrite* settlement increased with age up to day 3, but, conversely, that settlement success declined from day 4 onwards. Toonen and Pawlik (1994) disputed the 'desperate larva' hypothesis, observing that the majority of *Hydroides dianthus* larvae settled soon after reaching competence, ca. 7- 8 days post fertilisation, with settlement continuing, but in low numbers only, up to day 26. The *E. modestus* settlement behaviour combined both of these strategies. While young cyprids followed the Toonen and Pawlik polychaete observations, settlement by old cyprids was consistent with the Wilson/ Knight-Jones hypothesis. Two hypotheses are proposed to explain *E. modestus* behaviour; the first considers a single larval type with different behaviour dependent on age, and the second considers two distinct larval types that have peak settlement at different ages. Figure 4.7 illustrates these hypotheses.

The first hypothesis considers a single larval type with three stages of activity:- 1) 'Decisive' larvae : Young *E. modestus* cyprids (Day 0 and Day 1) are 'decisive'; they decide if conditions are favourable, or not, and settle only if they are i.e. as in assay wells treated with SF. Larvae are competent with an instinctive drive (sensu Crisp and Meadows, 1963) to settle and settlement is then brought about by environmental cues; 2) 'Deferring' larvae: Some mid-aged cyprids (Day 2 – Day 4) do settle, but many defer settlement. The instinctive drive may remain and behaviour may be explained by a period of habituation (e.g. Cohen et al., 1997; Rankin and Rose, 2001); 3) 'Desperate' larvae: Cyprids resume discriminatory settlement behaviour, but with increasing age they become 'desperate'; they settle readily and become increasingly less discriminating with increasing age as energy reserves are depleted, i.e. as indicated by increasing levels of control settlement with age.

(a)




(b)

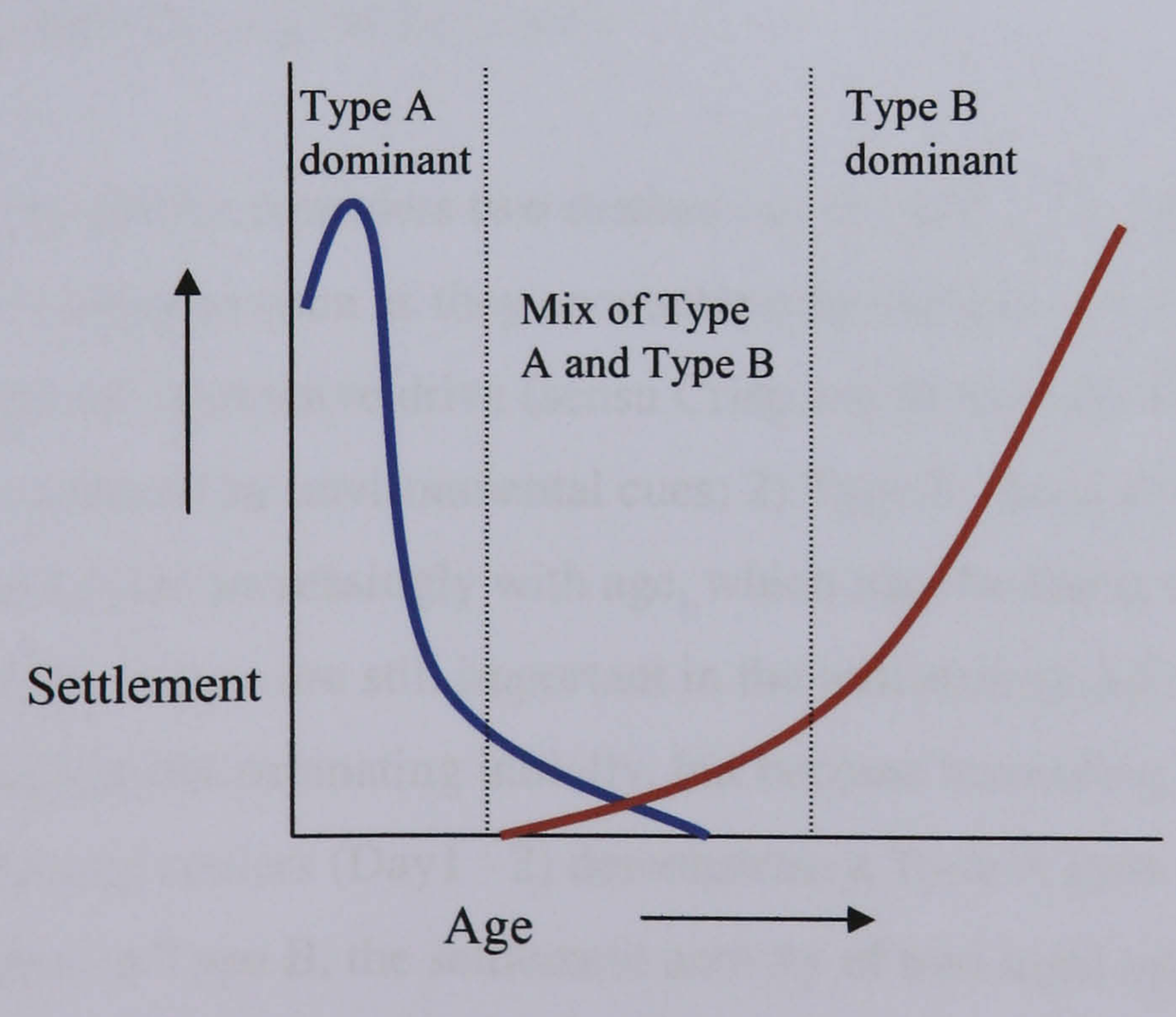



Figure 4.7: Diagrams illustrating two different hypotheses to explain settlement behaviour of *E. modestus* in response to conspecific SF; (a) Hypothesis 1: one larval type and (b) Hypothesis 2: two larval types.

Juvenile barnacles from 'decisive' settlers, starting life with a greater energy reserve, have improved early feeding capability and initial growth (Pechenik et al., 1993). Survival of individuals may be improved both directly, and also by giving them a competitive advantage. Assay observations suggested that 'deferment' was accompanied by a suspension of searching; many mid-aged larvae were motionless for extended periods with only short intermittent periods of swimming. The highly active swimming as observed with Day 0 larvae was absent. If searching were suspended in the natural environment, it would conserve energy reserves of individuals, while allowing them to be relocated by stochastic forces. Thus, the competent period of settlement may be extended, and the chance of finding the suitable habitat improved. However, it could be equally true that a 'deferring' larva may miss an ideal habitat. Additionally, this behaviour may enable a greater dispersal of a single cohort, which could alleviate dense settlement and reduce intraspecific competition. Conversely, postponement of settlement may adversely affect settlement capability and juvenile growth rates (Pechenik et al., 1993). The less-discriminating older larvae settle more readily and attempt adult life even if conditions may be far from ideal.

The second hypothesis considers two distinct larval types: - 1) Type A (Early settlers): Larvae settle readily as soon as they encounter a favourable environment. Larvae are competent with an instinctive drive (sensu Crisp and Meadows, 1963) to settle. Settlement is induced by environmental cues; 2) Type B (Late settlers): Larvae defer settlement, and settle increasingly with age, which may be due to a diminishing energy reserve. Settlement cues are still important in the initiation of settlement, with Type B individuals highly discriminating initially, but become increasing less so as they age. Thus, while young settlers (Day1 - 2) demonstrate a Type A strategy and old settlers (Day 5 onwards) a Type B, the settlement activity of mid-aged cyprids may be explained by a mix of the two types; while Type A activity is tailing off, Type B activity starts. The presence of two larval types may give the species a competitive advantage in much the same way as the proposed three stage single type. Type A early settlers have improved fitness, while Type B late settlers have the opportunity for greater dispersal.

An intermediate Type A and B mix ensures some settlement occurs throughout the period of competence.

An advantage of a single-type cyprid is that all individuals have the same settlement opportunities. The disadvantages are not only that 'deferring' larvae that delay metamorphosis may miss a suitable habitat, but also that extended pelagic existence would increase the chance of predation and may be more costly in terms of energy, which could be deleterious to less fit individuals (Crisp, 1974; Pechenik et al., 1993). The advantage for cyprids of the 'two-type' hypothesis is that energy needs are focused into a single phase of activity, while the disadvantage is that each type relies on being in the right place at the right time, and this could lead to higher pre-settlement mortality. Alternatively, Type B settlers may respond to a different cue, such that the presence of the alternative would induce early settlement. Two behavioural types have been described for the species *H. dianthus* (Toonen and Pawlik, 1994). All individuals were capable of early settlement, but each type responded to different environmental cues, one to the presence of conspecifics, the other to biofilm, and thus, in a single cue assay, it appeared that some individuals delayed settlement. This could also explain *E. modestus* behaviour and a separate investigation would be useful, though a cue other than biofilm may be implicated. Krug (2001) described the 'bet-hedging' strategy of a Californian population of the opisthobranch mollusc *Alderia modesta*. Adults produce two larval types with differing settlement behaviour; the first settles spontaneously, while the second delays metamorphosis until encountering a chemical cue from the adult host and obligate food source, *Vaucheria longicaulis*. These polymorphisms enable greater dispersal from a single clutch.

Variation in SF-induced settlement occurred between cohorts, for example Day 2/Day 3 settlement varied from 0 to >20%. Phenotypic plasticity is the capacity of a single given genotype to amend behaviour (Krug, 2001), or produce different morphologies (Li and Denny, 2004), in response to the environment in order to improve individual survival. Krug (2001) noted that larvae of starved *A. modesta* adults were more likely to produce larvae that delayed metamorphosis until encountering a cue from the food source. The

effect of the adult condition on cyprid behaviour is unknown, but may have been an influence. Additionally, some cyprids settle irrespective of assay conditions, which is difficult to attribute to either hypothesis. This behaviour could be explained by the presence of a separate 'pioneer' type (Crisp, 1974). Conversely, some cyprids fail to settle. Both behaviours are consistent with either hypothesis.

**Nitrocellulose assays**

The 50 and 100 $\mu$g spot$^{-1}$ assays with 400 cyprids in 400 ml and buffer only controls, gave a significant outcome at 50 $\mu$g spot$^{-1}$ and all further nitrocellulose assays with SF were carried out at this concentration. This concentration has also been used successfully with *B. amphitrite* and *S. balanoides* cyprids (Matsumura et al., 2000).

## 4.4.2 *Semibalanus balanoides*

**Optimum temperature and concentration of SF**

Low settlement by *S. balanoides* cyprids may have reflected the use of 'young' larvae in assays. Cyprids were used the day after collection, and the results of the assays may have been improved by laboratory ageing. Crisp and Meadows (1963) showed that while 30% of newly collected cyprids settled within 24 hours, settlement increased to more than 60% by ageing in glass jars for 5 days. However, while age may have contributed to low settlement at the start of the assays, cyprids had the opportunity to age during the extended assay duration of 21 days. Even so, settlement after 21 days was less than 10% at 19°C, and less than 1% at all other temperatures in both sets of assays. The earlier incidence of settlement in the later assays, as well as the earlier incidence of final settlement, may indicate that the mean cyprid age in the second collection was older than in the first. However, settlement in both assay sets was otherwise comparable, suggesting that the larvae were of comparable age and other factors may have contributed to low settlement. The assay design may have been fundamentally flawed. The assay method, used by Crisp and Meadows (1963) and Larman and Gabbott (1975), incorporated flowing water. However, although this may influence initial attachment (Crisp and Knight-Jones, 1953), Crisp and Meadows (1963) noted that it had little

positive effect if the surface was unfavourable with larvae preferring slate to 'Tufnol' surfaces. Successful settlement on a substratum is dependent on adhesion, a surface physico-chemical phenomenon (Wiegemann, 2005). Hills and Thomason (1998) determined that settlement on tiles prepared with a surface impression of 0.5 mm sand grains was 15 times greater than on smooth tiles of the same material (3 cyprids $cm^{-2}$ compared to 0.2 cyprids $cm^{-2}$). Thus, the smoothness of the polystyrene surface, as well as its 'wetability' determined from its surface free energy, may have reduced settlement by this species.

The optimal temperature for settlement was similar to that of early *S. balanoides* laboratory experiments that were carried out at room temperature (i.e. probably ca. 20-23°C) (e.g. Crisp and Meadows, 1962, 1963; Larman and Gabbott, 1975; Barnett and Crisp, 1979). However, more recently assays at 6°C have been successfully employed for *S. balanoides* (Matsumura et al., 2000), suggesting that larvae may be competent across a wide temperature range, and similar to geographic variation experienced in the natural environment.

**Nitrocellulose membrane trial assays**

Although this method had been used successfully by Matsumura et al. (2000) at a temperature of 6°C, it was decided to carry out the assays at 19°C, the optimal temperature of the 24-well plate assays. The cyprids were aged for 5 days, but settlement in both assays was low, and much lower than reported by Matsumura et al. (2000). In this earlier research, cyprids were aged for 6 days, and the same concentration of conspecific SF (50 $\mu g$ $spot^{-1}$) was used. Mean settlement of ca. 8 cyprids $spot^{-1}$ after 48 hours was recorded. In the present study, settlement did not occur until day 4 in the 100 $\mu g$ $spot^{-1}$ assay and day 6 in the 50 $\mu g$ $spot^{-1}$ assay, and then mean settlement $spot^{-1}$ was ca. 1 cyprid. While this may suggest that 6°C was a more effective temperature for the investigation, other factors such as physiological age of wild cyprids may account for the variation. Additionally, there were differences in the assay design.

## 4.5 Conclusion

The results of the experiments suggested the use of *E. modestus,* rather than *S. balanoides,* as a model species for settlement behaviour experiments, although its use may be limited to experiments using SF. *E. modestus* larvae were used effectively in both the 24-well plate and the nitrocellulose membrane assays despite floating behaviour by cyprids, and an assay temperature of 22±1°C, the same as that of larval culture, was indicated. In 24-well plate assays, settlement in response to SF was significantly higher than controls at an SF concentration of 10 $\mu$g ml$^{-1}$, the same as *B. amphitrite* (Matsumura et al., 1998). In nitrocellulose assays with blank controls, settlement was significantly higher than controls at a SF concentration of 50 $\mu$g ml$^{-1}$, again similar to *B. amphitrite* (Matsumura et al., 1998a and 2000).

# Chapter 5

# The settlement-inducing protein complex of *Elminius modestus*

# 5.1 Introduction

Several barnacle species contain SIPC-like proteins (Kato-Yoshinaga et al., 2000; Clare and Matsumura, 2000). Settlement by barnacles in response to allospecific cues suggests that different SIPCs are either sufficiently similar to be recognised by several species, or else that the SIPC receptor is broadly tuned (Clare and Matsumura, 2000). The SIPC of *B. amphitrite* has been isolated (Matsumura et al., 1998a) and the primary structure identified using molecular techniques (A. Clare pers.comm.). However, knowledge of the full cDNA sequence of the *B. amphitrite* SIPC has not enabled the isolation of SIPC from other barnacle species (R. Kirby pers.comm.). The essential next step is to determine the amino acid sequence of a corresponding gene from another related species. This will help identify conserved areas of the gene that may assist primer design and the isolation of SIPCs from other barnacle species. *E. modestus* is a suitable second species, as it has an appropriately-distant phylogeny to *B. amphitrite* (Pérez-Losada et al., 2004) and is amenable to laboratory study. The aim of this research was to isolate the SIPC of *E.modestus* and determine part of its sequence.

**Experimental objectives**

A. To isolate the SIPC of *E. modestus* and obtain partial amino acid sequences.
B. To design degenerate oligonucleotide primers from the partial SIPC amino acid sequence(s).
C. To determine using molecular biological techniques the primary sequence of the selected subunit of *E. modestus* SIPC.

# 5.2 Materials and methods

## Protein purification and subunit isolation

### Subunit identification

Using the crude protein samples prepared for con- and allo-specific settlement experiments (6.2), 20 $\mu$g total protein of *B. amphitrite* and *E. modestus* in Laemmli buffer were electrophoresed on a 10% acrylamide SDS-PAGE minigel (2.4.1.2.1). Molecular weight markers (Sigma high range markers) were used to the left and right of the protein samples. The gel was then used for western blotting (2.4.1.2.3) and the protein lanes were transferred to PVDF membrane. The left and right areas of the PVDF membrane, where the marker lanes were situated, were removed and stained separately with Coomassie Brilliant Blue, while the main membrane was immunostained using the anti-76 kDa *B. amphitrite* antibody. After destaining, the PVDF membrane was reconstructed carefully and attached to a sheet of card. The molecular weights of the immunostained bands were estimated against the marker lanes using the Syngene Gene Tools computer package.

### Subunit isolation

An SIPC-like protein was isolated from *E. modestus* adult barnacles (2.4.2) and a band equivalent to the 76 kDa subunit of *B. amphitrite* SIPC was selected. Four separate samples were prepared for amino acid sequencing by Edman degradation. The samples were prepared from four preparations of concentrated crude SF extracted from different batches of *E. modestus* adults.

### Gene isolation

Two samples of cDNA were prepared (2.5.1) from *E. modestus* adults maintained in an ambient aquarium (the first for four days and the second for less than 24 hours) using Superscript II RNase H- Reverse Transcriptase and Thermoscript RNase H- Reverse Transcriptase (Invitrogen, UK) respectively. The two *E. modestus* SIPC amino acid sequences determined by Edman degradation were aligned with the full-length amino

acid sequence of *B. amphitrite* SIPC to determine their approximate sequence position. Degenerate nested primers were designed to each amino acid sequence. The *E. modestus* primer reference name was designated as follows:-

EM, followed by A for Sequence 1 or Q for Sequence 2 – followed by F, for forward or R for reverse, followed by a number.

The *E. modestus* degenerate primers and available *B. amphitrite* 76 kDa degenerate primers (C.Dreanno pers.comm.) were used in PCR amplifications. Isolation of sequence from the gene was attempted by 2-step nested PCR as follows:- 1) *E. modestus* degenerate primers were used to isolate a ca. 150 bp sequence; 2) A combination of *E. modestus* and *B. amphitrite* degenerate primers were used to give PCR products of varying sizes. These experiments were carried out using all possible combinations of first round primers, followed by nested PCR, using all possible nested combinations; and 3) A 3' RACE was attempted on a dT adapted cDNA with *E. modestus* degenerate 5' primers and a 3' dT-adapter primer (ca. 2500 bp). Standard PCR reactions (2.5.2) were performed, followed by a series of modified PCR experiments, in which cDNA concentration, annealing temperature and extension times were varied (2.5.2.4).

## 5.3 Results

Figure 5.1 shows the isolation strategy for *E. modestus* SIPC and its gene, and summarises the results.

### Protein isolation and purification

Immunostaining produced a single band for the *B. amphitrite* sample, and two bands for the *E. modestus* sample. The upper *E. modestus* band migrated to a position similar to that of *B. amphitrite*. Figure 5.2 shows the immunostaining result with details of the estimated molecular mass of the bands. The *E. modestus* 93.5 kDa subunit was selected for sequencing. Table 5.1 summarises the results of the four preparations of concentrated SF. The protein content of the crude and concentrated SF $g^{-1}$ of starting

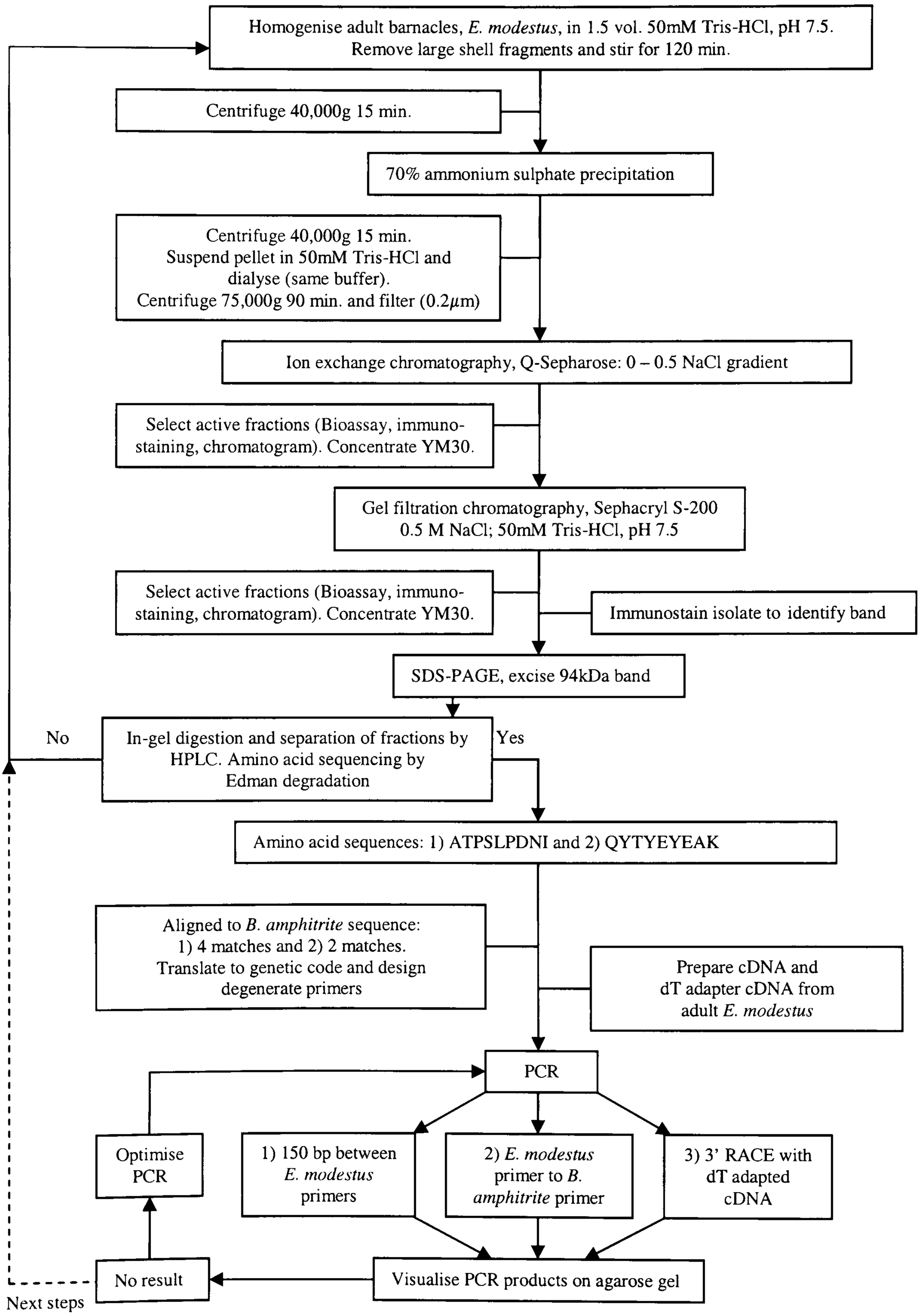



Figure 5.1: Isolation of *E. modestus* SIPC and attempted gene isolation

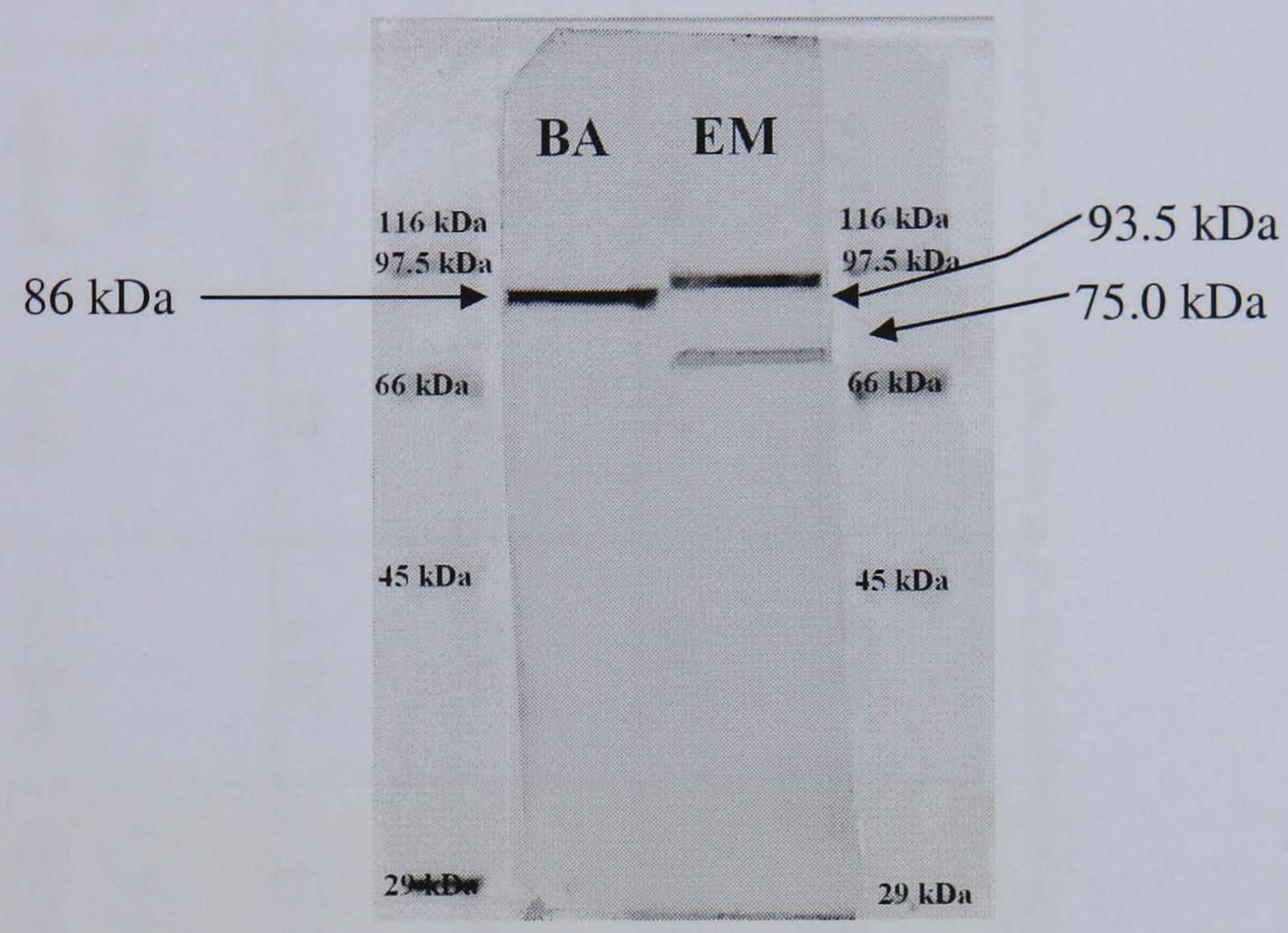



Figure 5.2: Immunostained *B. amphitrite* and *E. modestus* SF using the anti - 76 kDa *B. amphitrite* SIPC antibody

| Preparation | Wet weight of barnacles* (g) in buffer(ml) | Protein content of crude Settlement Factor | | | | Protein content of concentrated crude Settlement Factor | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein (mg/ml) | Volume (ml) | Total protein (mg) | Protein/g barnacle (mg $g^{-1}$) | Protein (mg/ml) | Volume (ml) | Total protein (mg) | Protein/ crude extract (%) | Protein/ g barnacle (mg $g^{-1}$) |
| 1. Prepared January 2002* | 600 1000 | 3.23 | 975 | 3149.2 | 5.25 | 31.7 | 41 | 1299.7 | 41 | 2.16 |
| 2. Prepared February 2002 | 200 400 | 1.58 | 365 | 576.70 | 2.88 | 15.7 | 26 | 408.2 | 71 | 2.04 |
| 3. Prepared July 2002 | 200 300 | 3.63 | 290 | 1052.7 | 5.26 | 13.9 | 29 | 403.1 | 38 | 2.01 |
| 4. Prepared March 2003 | 250 400 | 2.71 | 360 | 975.6 | 3.90 | 17.1 | 36 | 615.6 | 63 | 2.46 |

* Sample 1, January 2002, was prepared primarily for use in assays with a proportion of it set aside for purification of SIPC.

Table 5.1: Summary results of the protein extraction from adult *E. modestus*

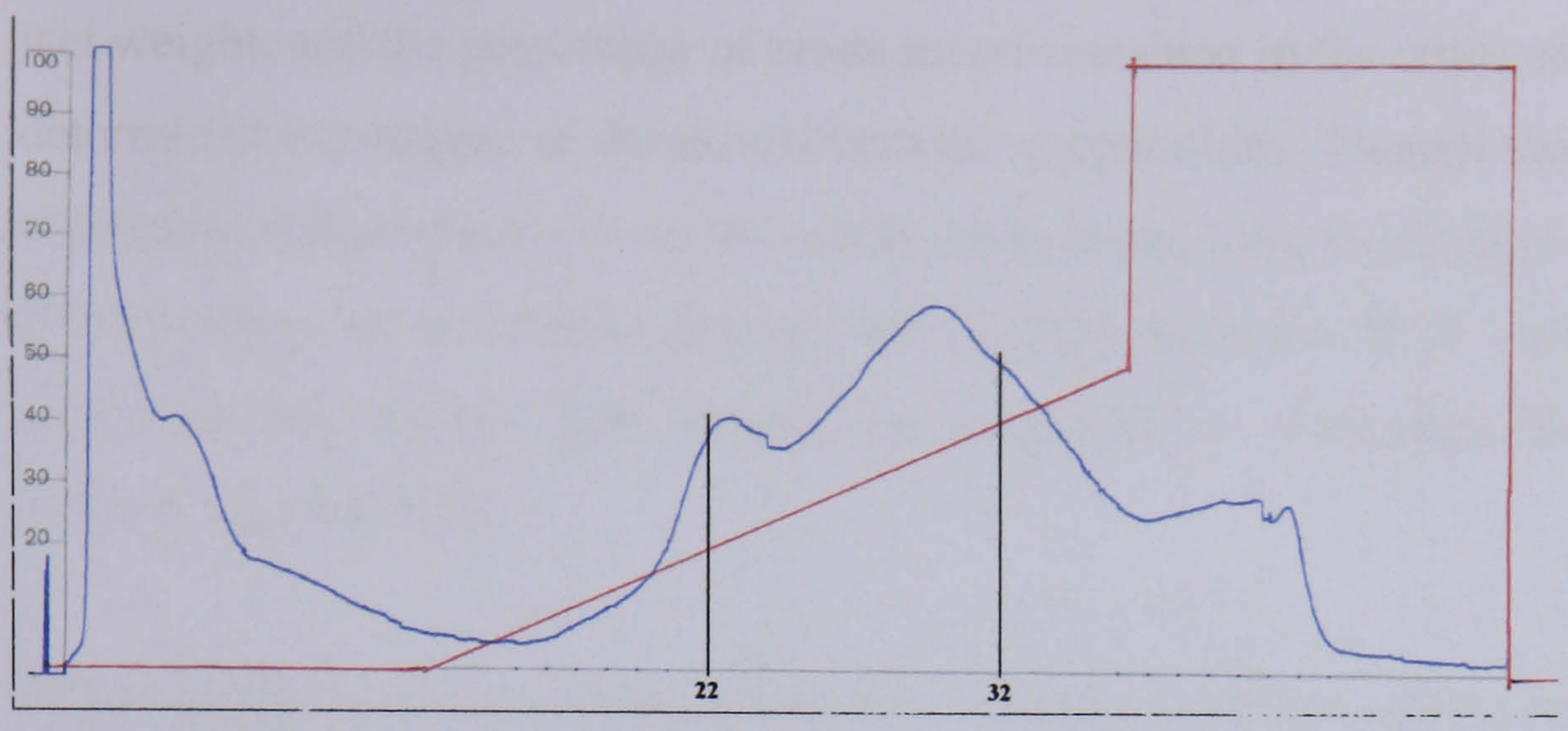



Figure 5.3: Ion exchange chromatograph of the elution of a sample of *E. modestus* SF.

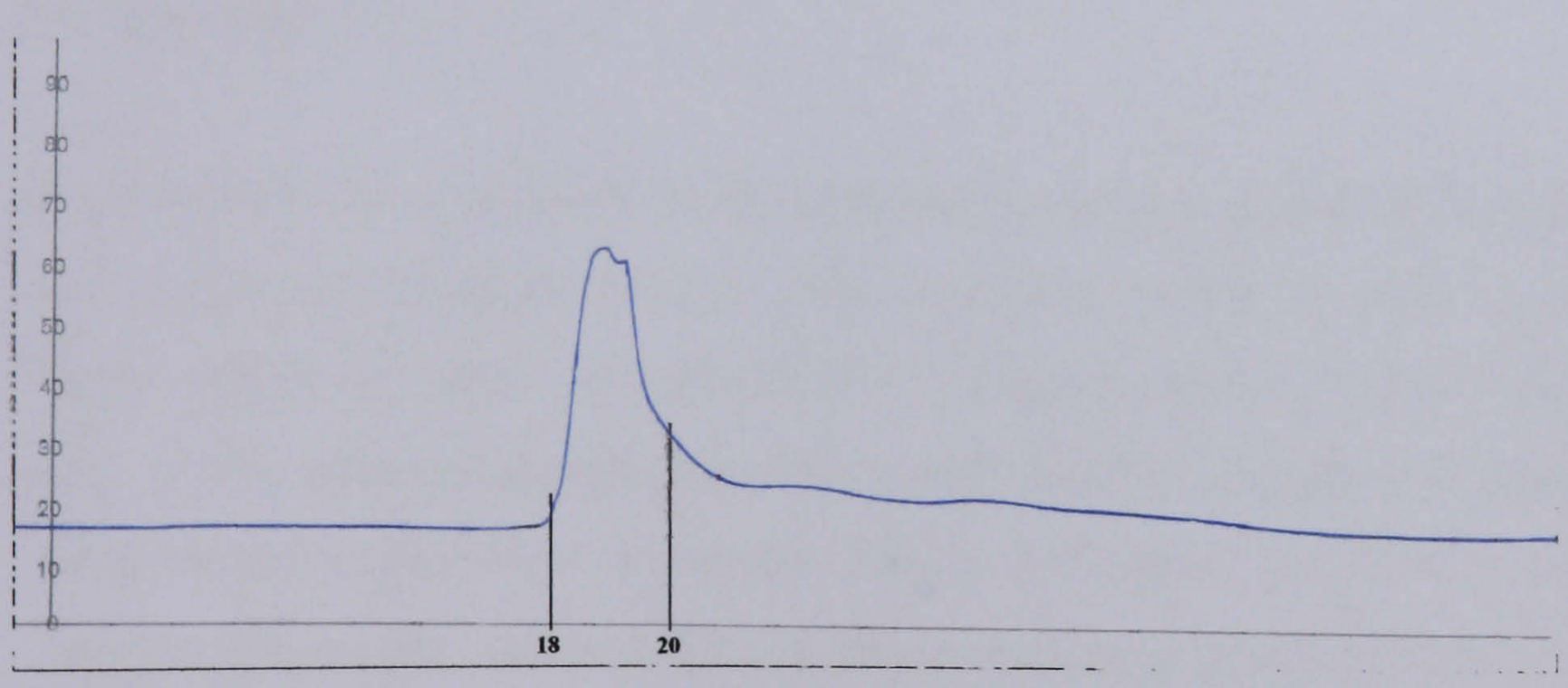



Figure 5.4: Gel filtration chromatogram of the elution of a sample of reconcentrated 'active' ion-exchange fractions.

wet weight, and the percentage of crude protein retained in the crude sample, were determined to compare of the results between preparations. Though there were some noticeable differences between the initial crude extractions, the final protein content $g^{-1}$ of barnacle tissue was similar for all batches of concentrated SF (2 – 2.5 mg $g^{-1}$ wet weight barnacle tissue). The outcome was comparable to extractions from different barnacle species (6.3).

The total protein content of the SF sample used for each ion-exchange elution was similar (100 – 110 mg) and the protein content of samples after purification was equivalent. Figure 5.3 shows a typical ion exchange chromatogram with fractions selected for further purification indicated. Fractions 22 to 32, selected by a combination of settlement bioassays and immunostaining results, were considered active, i.e. containing SIPC, and were purified further. Figure 5.4 shows a typical gel filtration chromatogram, with fractions selected for further purification indicated. Fractions 18 to 20, selected primarily by settlement bioassay and confirmed by immunostaining, were purified further.

Three subunits were detected by electrophoresis of the purified SIPC, a situation similar to *B. amphitrite* (Matsumura et al., 1998a). However, when the sample was concentrated further, additional bands were also visible, indicating that the sample was not 100% pure. As the additional bands were only visible when the sample was concentrated, the sample was dominated by one protein. The 93.5 kDa band was intense in the concentrated sample, and an SDS-PAGE exponential gradient gel (2.4.1.2.2) indicated that this band comprised two bands with estimated molecular masses of 94 kDa and 93 kDa. These bands, and neighbouring bands, were each cut out from three gel lanes. The excised bands were homogenized separately in small amounts of Laemmli buffer and applied to separate lanes of a 10% gel and electrophoresed. Western blotting using the anti-76 kDa *B. amphitrite* antibody detected bands of 94, 93 and 75 kDa. The 94 kDa subunit was selected for sequencing.

The isolation procedures were repeated and excised bands pooled resulting in four samples: - 1) 5 bands, 2) 27 bands, 3) 54 bands and 4) 113 bands. Amino acid sequencing of Sample 1 was unsuccessful, suggesting the protein was likely to be n-terminally blocked. All other samples were in-gel digested with the fragments separated by high pressure liquid chromatography (HPLC) prior to sequencing by Edman degradation. The sequencing attempts of Sample 2 and Sample 3 were also unsuccessful. The samples were digested with endoproteinase Lys-C, which cleaves at the carboxyl side of lysine. The protein content of the fragments was disproportionately small compared to the quantity of the subunit sample (J. Gray pers. comm.), indicating that the digestion was incomplete. Additionally, the Lys-C proteinase, produced many small fragments that were difficult to resolve by HPLC and resulted in a heavy baseline 'noise' when sequenced (J. Gray pers. comm.). The digestion of the fourth sample was achieved using endoproteinase Glu-C (V8), which cleaves at the carboxyl side of both glutamic acid and aspartic acid. The digested yield was again disproportionately small compared to the sample size, but sufficient fragmented sample was obtained for sequencing. Fragments were larger than those digested with Lys-C, though the HPLC resolution was hindered by the presence of similar sized fragments that were difficult to separate (J. Gray, G. Kemp pers.comms).

Two short amino acid sequences were obtained: 1) ATPSLPDNI and 2) QYTYEYEAK. The sequences were aligned to the 76 kDa *B. amphitrite* amino acid sequence as follows:

Sequence 1: *B. amphitrite* amino acids position 858 – 867 of the 76 kDa subunit

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *B. amphitrite* | V | T | S | E | M | P | D | T | I |
| *E. modestus* | A | T | P | S | L | P | D | N | I |

Sequence 2: *Balanus amphitrite* amino acids position 899 – 908 of the 76 kDa subunit

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *B. amphitrite* | E | V | S | L | P | Y | S | M | K |
| *E. modestus* | Q | Y | T | Y | E | Y | E | A | K |

(C. Dreanno pers.comm)

The two sequences were closely positioned with 32 amino acids between the end of Sequence 1 and the start of Sequence 2.

**Gene isolation**

The $A_{260}/A_{280}$ ratios of total RNA were 1.81 and 1.97 for samples 1 and 2 respectively. The $A_{260}/A_{280}$ ratios of the mRNA obtained from these were 1.76 and 1.93 for samples 1 and 2 respectively. The amount of mRNA obtained from the total RNA, i.e. mRNA expressed as a percentage of total RNA, was lower for sample 1 (1.10%) than sample 2 (3.29%).

Figures 5.5 and 5.6 illustrate the amino acid sequences, their corresponding forward and reverse translated DNA sequences with the sequence selections for the primers shown. Degeneracy and the approximate primer melting temperatures are also indicated. The choice of primers was limited by the amino acid sequence information available and the degeneracy of Sequence 1 was particularly high. Figure 5.7 illustrates the position of all available *B. amphitrite* degenerate primers, and the location of the *E. modestus* primers within the sequence. For first round PCR, two forward *B. amphitrite* primers, BA-F2 and BA-F3, were used with EMA and EMQ series reverse primers. Two reverse *B. amphitrite* primers, BA-R1 and BA-R2, were used with EMA and EMQ series forward primers. The *B. amphitrite* primers provided greater opportunity for nested PCR.

All PCR experiments had a negative outcome. Experiments to isolate the 150 bp of sequence between the *E. modestus* primers had no visible PCR products on 3% gels for either the first round or nested PCR. Experiments using *E. modestus* and *B. amphitrite* degenerate primers similarly gave no visible first round PCR products on 1% gels and only smeared DNA was observed from nested PCR. The 3' RACE experiments with the dT-adapter primer and E. *modestus* degenerate primer also gave no visible first round PCR products on 1% gels, and inappropriately sized bands and smeared DNA upon nested PCR.

(a)

| Amino acids | A | T | P | S | L | P | D | N | I |
|---|---|---|---|---|---|---|---|---|---|

(b)

| Genetic code | G | C | N | A | C | N | C | C | N | W | S | N | Y | T | N | C | C | N | G | A | Y | A | A | Y | A | T | H | Degen -eracy | Tm (°C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EMA-F1 | | | | A | C | i | C | C | N | W | S | N | Y | T | N | C | C | i | G | A | Y | A | A | | | | | 2304 | 54.5 |
| EMA-F2 | | | | | | | | C | N | W | S | N | Y | T | N | C | C | N | G | A | Y | A | A | Y | A | T | | 4608 | 47.5 |

(c)

| Genetic code | D | A | T | R | T | T | R | T | C | N | G | G | N | A | R | N | S | W | N | G | G | N | G | T | N | G | C | Degen -eracy | Tm (°C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EMA-R1 | | A | T | R | T | T | R | T | C | N | G | G | i | A | R | N | S | W | N | G | | | | | | | | 4608 | 50.9 |
| EMA-R2 | | | | | T | T | R | T | C | N | G | G | N | A | R | i | S | W | N | G | G | N | G | T | | | | 4608 | 56.1 |

Figure 5.5: Forward and reverse degenerate primers designed from the amino acid sequence ATPSLPDNI; (a) Amino acid, (b) Genetic code forward: 5' – 3' and (c) Genetic code reversed: 5' – 3'

(a)

| Amino acids | Q | Y | T | Y | E | Y | E | A | K |
|---|---|---|---|---|---|---|---|---|---|

(b)

| Genetic code | C | A | R | T | A | Y | A | C | N | T | A | Y | G | A | R | T | A | Y | G | A | R | G | C | N | A | A | R | Degen-eracy | Tm (°C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EMQ-F1 | C | A | R | T | A | Y | A | C | N | T | A | Y | G | A | R | T | A | Y | G | A | | | | | | | | 128 | 43.8 |
| EMQ-F | | | | T | A | Y | A | C | N | T | A | Y | G | A | R | T | A | Y | G | A | R | G | | | | | | 128 | 41.2 |
| EMA-F2 | | | | | | | A | C | N | T | A | Y | G | A | R | T | A | Y | G | A | R | G | C | N | A | | | 256 | 47.9 |

(c)

| Genetic code | Y | T | T | N | G | C | Y | T | C | R | T | A | Y | T | C | R | T | A | N | G | T | R | T | A | Y | T | G | Degen-eracy | Tm (°C) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EMQ-R1 | | | T | N | G | C | Y | T | C | R | T | A | Y | T | C | R | T | A | N | G | T | | | | | | | 128 | 48.8 |
| EMQ-R | | | | | | C | Y | T | C | R | T | A | Y | T | C | R | T | A | N | G | T | R | T | A | | | | 256 | 43.2 |
| EMQ-R2 | | | | | | | | T | C | R | T | A | Y | T | C | R | T | A | N | G | T | R | T | A | Y | T | G | 128 | 46.1 |

Figure 5.6: Forward and reverse degenerate primers designed from the amino acid sequence QYTYEYEAK; (a) Forward: 5' – 3' and (b) Reverse: 5' – 3'

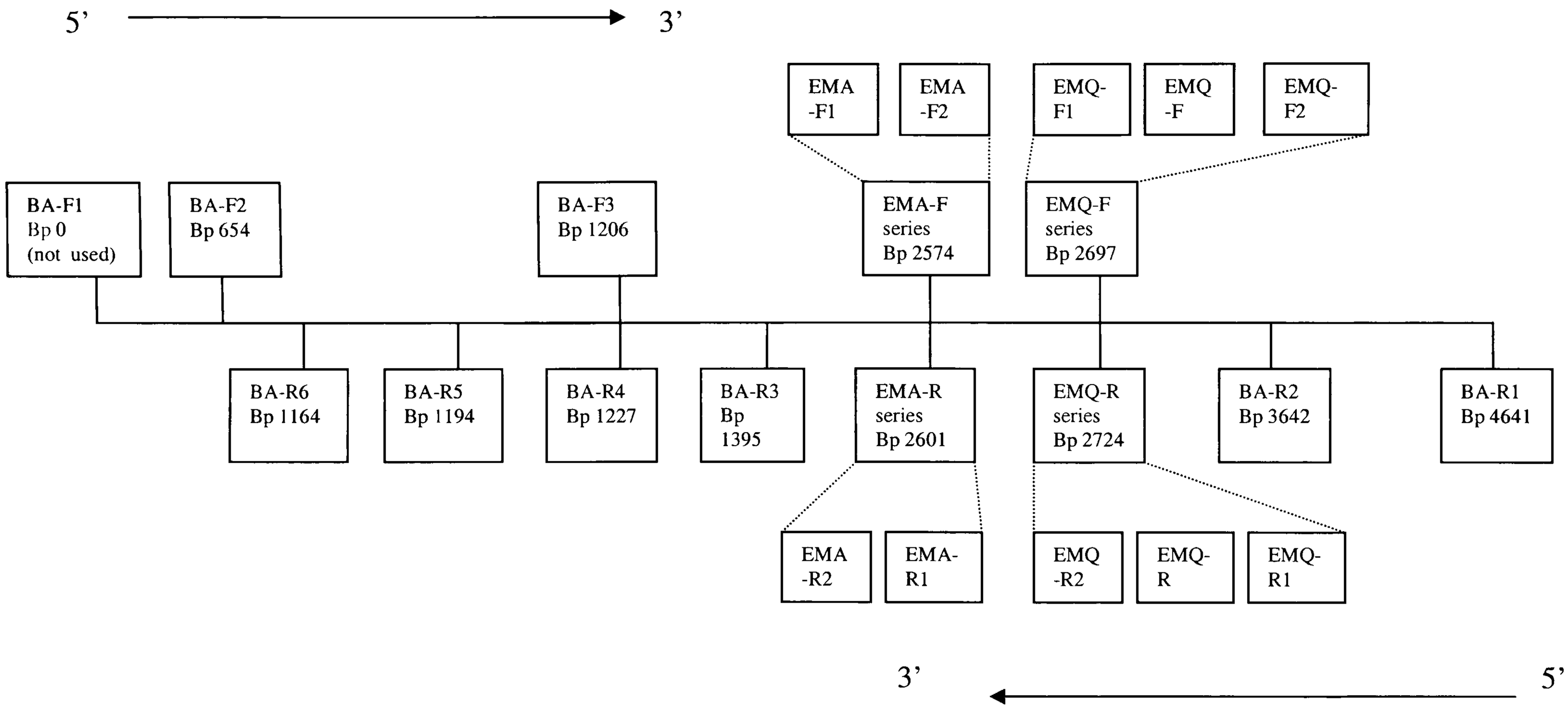



Figure 5.7: Line diagram illustrating the positions of available *B amphitrite* primers and *E modestus* primers within the 76 kDa subunit of *B. amphitrite* SIPC. The bp reference for each indicates the relevant position of the primers on the *B. amphitrite* 76 kDa SIPC subunit. The *B. amphitrite* primers (reference starting with BA) were developed during earlier research. The *E. modestus* primers (reference staring with EM) were developed from the known amino acid sequences.

## 5.4 Discussion

### SF preparation

Three separate antibodies had been raised against the *B. amphitrite* SIPC, with one for each of the three subunits, 76 kDa, 88 kDa and 98 kDa (Matsumura et al., 1998a). The anti-76 kDa antibody was selected as it had the strongest species-specific signal and high specificity that did not cross-react with other bands (Matsumura et al., 1998a). Additionally, it has been used successfully to detect SIPC-like glycoproteins in other barnacle species (Kato-Yoshinaga et al., 2000). Immunostaining of a sample of *E. modestus* SF after SDS-PAGE, indicated that two bands cross reacted with the anti-76 kDa *B. amphitrite* polyclonal antibody, one at a similar molecular mass to *B. amphitrite*, and a second lower molecular mass band. Thus, the *E. modestus* SF contained a SIPC-like protein, and it suggested that two of its subunits contain a shared epitope similar to that of *B. amphitrite*. The difference in the estimation of the *B. amphitrite* subunit compared to the original research (Matsumura et al., 1998a) may be due to the different molecular weight markers (Daiichi Pure Chemical Co, Japan). A possible source of error was the reattachment of the marker lanes, though care was taken to undertake this step accurately. Additionally, the method had the advantage of avoiding prestained markers that have a reputation of inaccuracy due to the affect of the pre-attached dye upon migration (Hames and Rickwood, 1998). While the presence of the two bands may indicate two separate subunits of similar size, the two bands were most likely dimers (Stryer, 1995); alternatively, if the lower band was due to degradation, the epitope was unaffected.

Despite differences between preparations in the total protein content of the extract after initial extractions from barnacle tissue, the protein content after ammonium sulphate precipitation was remarkably constant. The batches of *E. modestus* SF were prepared from barnacles collected at different times of year, and also different periods of laboratory culture. The first and second preparations were from the barnacles collected during December 2001, which were retained in an ambient temperature aquarium and fed *Artemia* sp. daily for 1 month and 2 months respectively. The reduced quantity of

the first protein extraction in the second preparation (2.88 mg $g^{-1}$ wet weight barnacles compared to 5.25 mg) may have indicated that that the physiological function of the barnacle had reduced considerably during their retention; the mRNA levels appear to decline rapidly in aquarium-kept barnacles (R. Kirby pers.comm.), resulting in reducing protein expression while retained. The third and fourth batches of SF were prepared from separate newly-collected barnacles that were retained for no more than 2 weeks. Thus, the variation in protein content after initial extraction (5.26 mg $g^{-1}$ wet weight barnacles July 2002 compared to 3.90 mg March 2003) is more likely to be attributable to seasonal variation. However, as the final protein content of all preparations was relatively uniform, certain proteins were apparently unaffected. Additionally, comparisons of the results of each further stage of SIPC isolation indicated the retention of similar protein quantities. As the SIPC-like protein was retained at each step, the content of this protein in all the adult barnacle collections may have been similar.

The ion exchange chromatogram (Figure 5.3) was similar to that of *B. amphitrite* (Matsumura et al., 1998a), and the gel filtration chromatograms of the two species were also similar (K. Matsumura pers.comm.). Additionally, the active *E. modestus* fractions after each of the chromatography steps corresponded to those of the *B. amphitrite* selections (K. Matsumura pers.comm.). Selection of active ion exchange fractions by bioassay was difficult, as cyprid settlement occurred on many of the protein areas. Cyprid settlement on fractions 2-10 may be explained by the likely presence of SIPC in the unattached protein that was washed from the column before the salt gradient, necessary for protein elution, was applied. Similarly, the final fractions, removed by the salt wash at the end of the elution, may also have contained SIPC. The bioassay of gel filtration fractions was more informative with settlement occurring on a limited number of fractions. *B. amphitrite* gregariousness is thought to be a response to a single glycoprotein, SIPC (Matsumura et al., 1998a). However, as the chromatographic isolation yielded a number of proteins, the selection of a SIPC-like subunit for sequencing was directed by western blotting with the available polyclonal antibody against the biologically-active 76 kDa subunit of *B. amphitrite* SIPC.

**Amino acid sequences**

The amino acid information obtained was limited to two short sequences, each 9 amino acids in length. The limited quantity of isolated fraction following protein digestion was considered central to the difficulty in obtaining longer sequences. The alignment of the *E. modestus* amino acid sequences to the primary *B. amphitrite* SIPC sequence suggested that the amino acids were part of the *E. modestus* SIPC. In addition to the direct matches (4 in Sequence 1 and 2 in Sequence 2), in each sequence 3 further aligned amino acids were separated by only one substitution in the genetic code. Additionally, a database search (BLAST) located a sequence fragment of the complement factor, a thioester containing protein of a central subsystem within innate immunity (Zipfel and Skerka, 1994; Nakao and Yano, 1998), of *Cyprinus carpio* (common carp) (Accession number AB007005.1) with a direct sequence match of six out of nine of the amino acids of Sequence 2. Thus, the *E. modestus* fragments were considered likely to be from SIPC.

The small disproportionate yield of digested fractions from the subunit sample suggested that the process may have been inhibited, although conversely the abundance of small peptides after subunit digestion suggested that the process proceeded too far. *B. amphitrite* SIPC belongs to a subgroup of the alpha-2-macroglobulins (A2M) (C. Dreanno pers.comm.), and due to the cross-reaction of the *B. amphitrite* polyclonal antibodies, it is likely that the SIPCs of all species are A2M-like. The difficulty in effective cleavage may be associated with the conservation of features of A2Ms, a protein of the innate immune system that has a broad-spectrum protease-binding function (Armstrong and Quigley, 1999), and therefore is resistant to protease digestion. However, the full sequence of *B. amphitrite* SIPC indicated that it does not have the thioester bond (C. Dreanno pers. comm.) that is present in all known A2Ms and is essential to the antiproteinase activity. Until the full sequences of additional barnacle species are established, the lack of a thioester bond cannot be assumed to be a common factor of SIPCs.

### PCR experiments

Many different combinations of parameters were tested in PCR experiments, though all were unsuccessful. The *E. modestus* primers were less than ideal due to the limited amino acid information. As the single most important factor leading to successful DNA amplification is the quality of the primers (R. Kirby pers.comm.), the chances of success were limited. Thus, the solution to the problem would seem not to lie within the fine tuning of PCR parameters, but in the redesign of primers from increased amino acid sequence information. A further attempt at protein isolation and sequencing is recommended. In view of the difficulty with the digestion of the *E. modestus* subunit, it may be provident to select an alternative barnacle species with the possibility that digestion is straightforward. A greater digested sample yield, in turn, could enable a longer amino acid sequence to be obtained.

## 5.5 Conclusion

Chromatography, SDS-PAGE and western blotting results suggest that an SIPC-like protein has been isolated from *E. modestus*. Although a small proportion of the primary sequence has been identified, the research is far from complete. A review of digestion parameters, or the use of an alternative species, may yield sufficient sequence for improved primer design.

# Chapter 6

# Laboratory experiments on con- and allo-specific settlement behaviour

## 6.1 Introduction

Earlier research has indicated that cyprids settle to both con- and allo-specific cues (Knight-Jones and Moyse, 1961; Larman and Gabbott, 1975; Barnett et al., 1979; Barnett and Crisp, 1979; Crisp, 1990; Whillis et al., 1990; Matsumura et al., 2000; Kato-Yoshinaga et al., 2000). Settlement specificity between species has been associated with systematic affinity (Knight-Jones, 1955; Crisp, 1990), i.e. the more closely related the species the greater the settlement response, although results have varied and a consensus has not been reached. The aim of this research was to further investigate con- and allo-specific settlement for *E. modestus* and *B. amphitrite* through single cue and multiple cue assays with cyprids of a known age. For single cue assays, cyprids of two ages from the same cohort were used in separate 24-well plate assays. Settlement behaviour to the selected SFs was investigated independently, but concurrently, and thus comparisons between results were possible. Multiple cue assays, using several SFs simultaneously, were undertaken by nitrocellulose membrane assay, such that cyprids were able to 'choose' between species. SFs were prepared for *E. modestus* and *B. amphitrite* and four other Balanomorpha, namely *B. crenatus, B. improvisus, C. montagui* and *S. balanoides*. The UK distribution of *E. modestus* is such that it interacts with all temperate species, while the distribution of *B. amphitrite* does not overlap with the other test species and *B. amphitrite* cyprids would not naturally encounter their SFs. The species of the selected SFs were chosen due to their different evolutionary relatedness (Pérez-Losada et al., 2004) to examine whether settlement accords to systematic affinity. Additionally, *B. amphitrite, B. crenatus* and *B. improvisus* are low shore and sublittoral species, *E. modestus* and *S. balanoides* are mid shore species and *C. montagui* is a high shore species.

**Experimental objectives**

A. To investigate settlement behaviour of *E. modestus* and *B. amphitrite* cypris larvae to con- and allo-specific cues in single cue assays.
B. To investigate settlement behaviour of *E.modestus* and *B. amphitrite* cypris larvae to con- and allo-specific cues in multiple cue 'choice' assays.

## 6.2 Materials and methods

### SF selection and preparation

SFs from six barnacle species were used in the experiments. The adult barnacles used for the preparation of the SF were as follows:- 1) *B. amphitrite,* transported alive from Shizuoko, Japan, removed from their substrate and stored at -80°C; 2) *B. crenatus,* collected from the Tamar Estuary, Devon, UK (50°23.3'N 4°12.2'W) on March 6th 2003 and held at 12°C for 1 week; 3) *B. improvisus,* collected from Tjarno Sweden during March 2003 and freeze-dried; 4) *C. montagui,* collected from Plymouth, Devon, UK (50°21.0'N 4°7.1'W) on March 5th 2003 and held at 12°C for 2 weeks; 4) *E. modestus,* collected from Great Bull Hill, Exmouth, Devon, UK (50°36.9'N 3°25.8'W) on March 4th 2003 and held at 12°C for 1 week; 5) *S. balanoides,* collected from Blackhall Rocks, Co. Durham, UK (54°44.5'N 1°16.1'E) on April 18th 2002. Each SF was prepared as described in Section 2.4.2.1. These single preparations of extracts were used for both the laboratory assays (this chapter) and the field experiments (Chapter 7).

The presence of SIPC, the active settlement protein in SF, was confirmed by immunostaining with a *B. amphitrite* SIPC polyclonal antibody. A total of 50 $\mu$g protein in Laemmli buffer of each sample was electrophoresed on a 10% acrylamide large gel and a western blot (2.4.1.2.3) was carried out using the anti-76 kDa polyclonal antibody to *B. amphitrite* SIPC.

### Experiments

### Experiment SF1 (Single cue assays)

Day 1 *E. modestus* and *B. amphitrite* cyprids from a single cohort of each species were used in separate 24-well plate assays (2.2.1). All *E. modestus* assays were carried out at 22°C, while *B. amphitrite* assays were carried out at 28°C. For both species, assays were set up using each of the six SFs. For each SF, six replicates of 4 concentrations of SF, 0.1, 1.0, 10.0 and 100 $\mu$g ml$^{-1}$, suspended in 0.45 $\mu$m filtered seawater, and six replicates

of a negative control, 0.45 $\mu$m filtered seawater, were prepared. Settlement was observed after 24 hours. Assays were repeated using Day 4 cyprids from the same cohorts. Then, all assays were repeated using a further larval cohort

**Experiment SF2 (Multiple cue assays)**

Day 0 *E. modestus* and Day 1 *B. amphitrite* cyprids from a single cohort of each species were used in separate 24-spot nitrocellulose membrane assays (2.2.2). The behaviour of each species was tested against their respective conspecific SFs, and selected allospecific SFs. All SFs were diluted in 50 mM Tris-HCl pH7.5 buffer to 50 $\mu$g ml$^{-1}$ and 1 ml aliquots were applied randomly to individual wells with 1 ml of 50 mM Tris-HCl pH7.5 buffer applied to remaining wells as a control. Three assays were set up simultaneously for each species. Two assays had 6 replicates of treatments as follows: 1) conspecific, *B. crenatus* and *C. montagui* SF, and the control treatment; 2) conspecific, *B. improvisus* and *S. balanoides* SF, and the control treatment. The third assay had 4 replicates each of the conspecific and 4 allospecific SFs, *B. crenatus, B. improvisus, C. montagui, S. balanoides*, and the control treatment. Settlement was observed after 24 hours.

**Experiment SF3 (Multiple cue assays)**

Parameters for Experiment SF3 were the same as experiment SF2 except that five assays were set up simultaneously for each species. Four assays had 6 replicates of treatments as follows: 1) conspecific, *B. crenatus* and *C. montagui* SFs, and the control; 2) conspecific, *B. improvisus* and *S. balanoides* SFs, and the control; 3) conspecific, *B. crenatus* and *B. improvisus* SFs, and the control; 4) conspecific, *C. montagui* and *S. balanoides* SFs, and the control. The fifth assay had 4 replicates each of the conspecific and the 4 allospecific SFs, and the control.

## 6.3 Results

### Preparation of SFs

Table 6.1 summarises the results of SF preparation for each of the six species. The first extraction of crude SFs from the different species had notably different protein gram$^{-1}$ of starting weight of barnacles, though the final protein content of three of the concentrated samples was similar; *B. amphitrite* was 2.20 mg g$^{-1}$ of barnacle tissue, *C. montagui* 2.10 mg, and *E. modestus* 2.16 mg. The *B. improvisus* extract, which was prepared from freeze-dried tissue, had a much higher concentration of protein at both extraction stages. The final concentration of the concentrated *S. balanoides* extract was more than 25% lower than that of the median value, though it was not the lowest at the initial extraction.

All SFs reacted to the anti-76 kDa polyclonal antibody of *B. amphitrite* SIPC (Figure 6.1). The immunostained bands were of different intensities. The *B. amphitrite* band was most intense, as would be expected of a species-specific antibody. *B. improvisus* was of a similar intensity, followed by *E. modestus* and *B. crenatus* with *E. modestus* having a more diffuse band than *B. crenatus*. The *S. balanoides* staining was less intense, and *C. montagui* the least. A second lower band was present in the *E. modestus* sample. Additionally, the position of the stained bands indicated different molecular masses for each sample.

### Experiment SF1 (Single cue 24-well assays)

### Day 1 cyprids

Settlement was comparable between cohorts for both species with ca. 50-60% at the preferred peak concentration. Figure 6.2 illustrates the 24-hour settlement for Day 1 cyprids of *B. amphitrite* and *E. modestus*. For all *E. modestus* assays, highest settlement occurred at a concentration of 10 $\mu$g ml$^{-1}$. Settlement was also highest at this concentration for the many of the *B. amphitrite* assays; exceptions were Cohort 2 with conspecific SF, Cohort 1 with *B. crenatus* SF, Cohorts 1 and 2 with *C. montagui* SF, where in each case highest settlement occurred at 100 $\mu$g ml$^{-1}$. For the majority of assays, *E. modestus* settlement at concentrations other than 10 $\mu$g ml$^{-1}$ was similar to the

| Species | Wet weight of barnacles* (g) in buffer (ml) | Protein content of crude Settlement Factor | | | | Protein content of concentrated crude Settlement Factor | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein (mg $ml^{-1}$) | Volume (ml) | Total protein (mg) | Protein/g barnacle (mg $g^{-1}$) | Protein (mg $ml^{-1}$) | Volume (ml) | Total protein (mg) | Protein/ crude extract (%) | Protein/ g barnacle (mg $g^{-1}$) |
| *B. amphitrite* | 230 g 400 ml | 1.61 | 340 | 547.4 | 2.38 | 17.5 | 29 | 507.5 | 93 | 2.20 |
| *B. crenatus* | 87 g 150 ml | 2.67 | 195 | 520.6 | 5.97 | 14.4 | 17 | 244.8 | 47 | 2.81 |
| *B. improvisus* | 122 g* 400 ml | 6.92 | 280 | 1937 | 15.88* | 23.4 | 43.8 | 1024.9 | 53 | 8.4* |
| *C. montagui* | 170 g 300 ml | 2.06 | 270 | 556.2 | 3.27 | 14.02 | 25.5 | 357.51 | 64 | 2.10 |
| *E. modestus* | 600 g 1000 ml | 3.23 | 975 | 3149.2 | 5.25 | 31.7 | 41 | 1299.7 | 41 | 2.16 |
| *S. balanoides* | 268 g 300 ml | 3.87 | 280 | 1083.6 | 4.04 | 14.0 | 31 | 434.0 | 40 | 1.62 |

Table 6.1: Results summary of protein extraction from six species of barnacles. * *B. improvisus* is freeze dried weight of barnacles

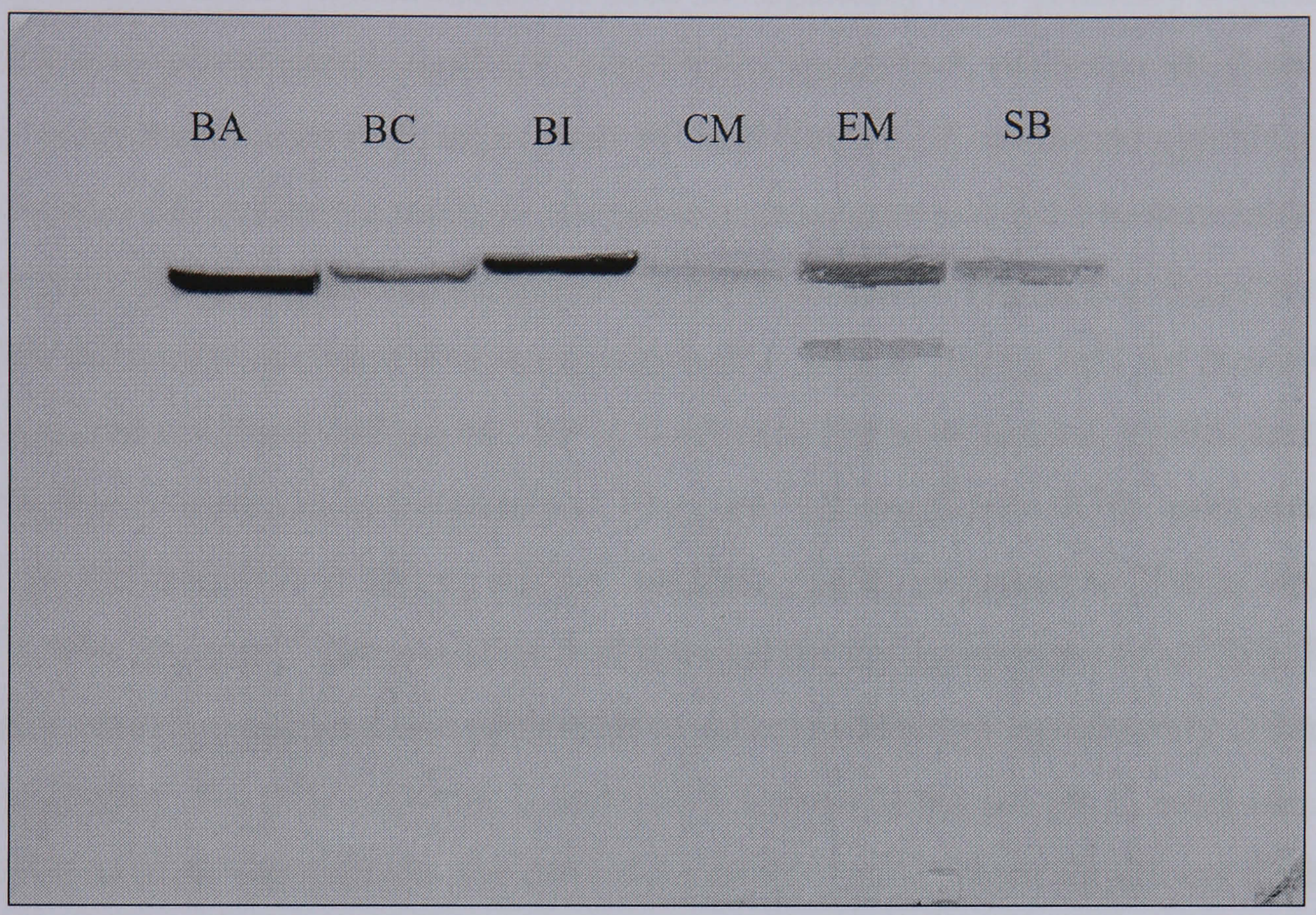



Figure 6.1: Photograph of PVDF membrane illustrating the results of immunostaining electrophoresed protein samples of SF from six barnacle species with the anti-76 kDa *B. amphitrite* antibody. From left to right: *B .amphitrite* (BA), *B. crenatus* (BC), *B. improvisus* (BI), *C. montagui* (CM), *E.modestus* (EM) and *S. balanoides* (SB).

control. In comparison, *B. amphitrite* settlement to the control and 0.01 $\mu$g ml$^{-1}$ SF were similar, though settlement at 1 $\mu$g ml$^{-1}$ was generally > 20%. Kruskal-Wallis analyses comparing different concentrations of each SF separately were generally significant ($P \leq 0.037$); exceptions were Cohort 1 *B. amphitrite* cyprids to *E. modestus* SF, both cohorts of *E. modestus* cyprids to *B. improvisus* SF, and Cohort 2 *E. modestus* cyprids to *S. balanoides* SF. A Dunn's multiple comparison procedure was used to determine where significant differences occurred and results are indicated in Figure 6.2. Significantly higher settlement occurred at concentrations of 1, 10 and 100 $\mu$g ml$^{-1}$ for *B.amphitrite* cyprids and at 10 and 100 $\mu$g ml$^{-1}$ for *E. modestus*. For both species, significantly higher settlement occurred most frequently at 10 $\mu$g ml$^{-1}$ (*B. amphitrite*, 83%, compared to 8% at 1 $\mu$g ml$^{-1}$ and 75% at 100 $\mu$g ml$^{-1}$; *E. modestus,* 66%, compared to 25% at 100 $\mu$g ml$^{-1}$). The 10 $\mu$g ml$^{-1}$ concentration was selected for the comparison of all SFs. Kruskal-Wallis statistics indicated that settlement to different SFs was significantly different for *B. amphitrite* $P = 0.018$, Cohort 1; and 0.005, Cohort 2) but not for *E. modestus*. A Dunn's multiple comparison was carried out to determine where significant differences occurred and results are indicated in Figure 6.3. For both species, while the majority of SF comparisons to the control were significantly different, comparisons between SFs were similar.

**Day 4 cyprids**

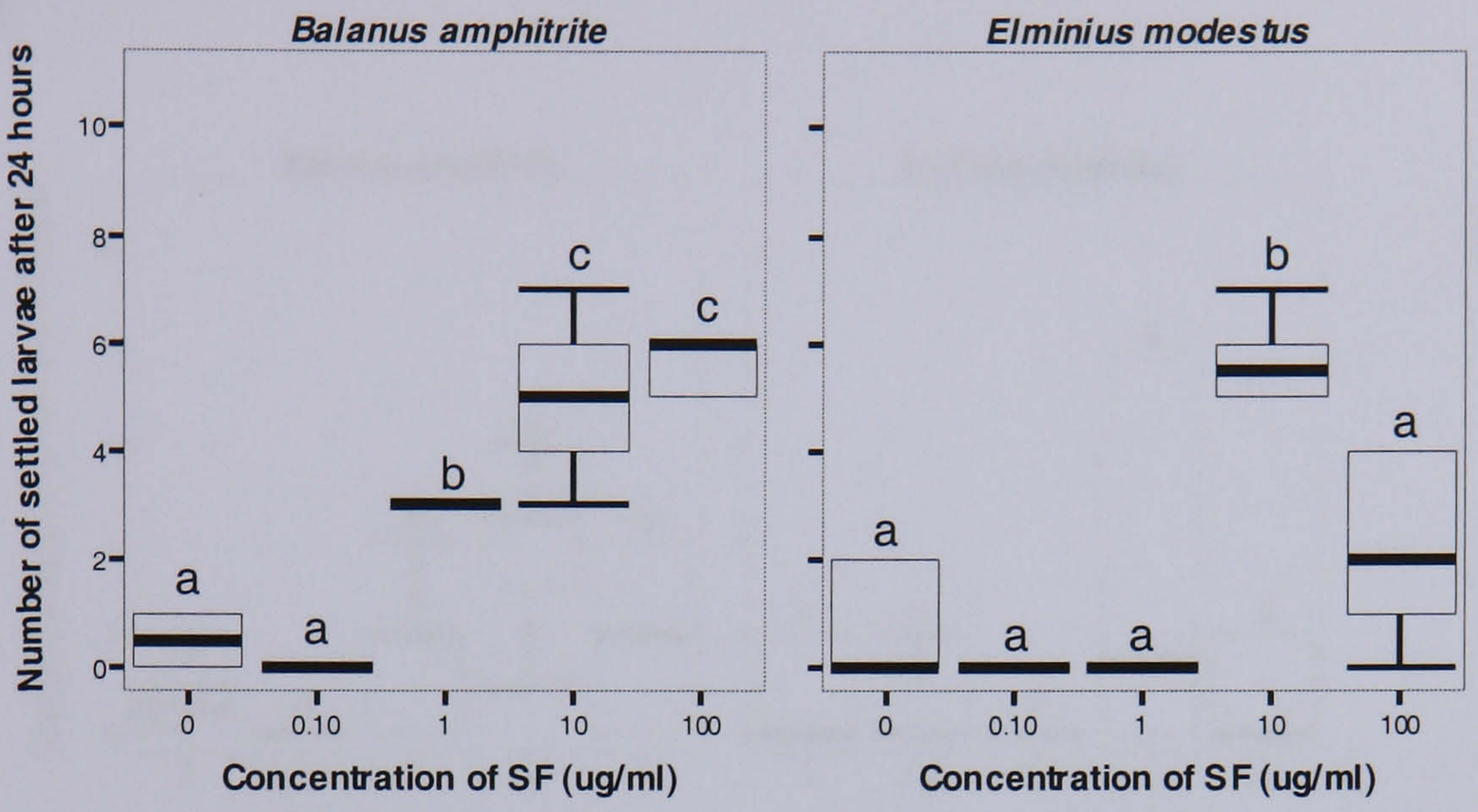
Peak mean settlement varied between 35-80% in *E. modestus* assays, and 35-90% in *B. amphitrite* assays, with more variation noted between assays and between cohorts than assays with Day 1 cyprids. Figure 6.4 shows 24-hour settlement for Day 4 cyprids of both species. All *E. modestus* assays had highest settlement at a concentration of 10 $\mu$g ml$^{-1}$. The majority of *B. amphitrite* assays had highest settlement at a concentration of 100 $\mu$g ml$^{-1}$; exceptions were Cohort 2 assays with *B. crenatus* SF and both cohorts with *E. modestus* and *S. balanoides* SFs. As for Day 1 cyprids, *E. modestus* settlement at all concentrations other than 10 $\mu$g ml$^{-1}$ continued to be similar to the control. Kruskal-Wallis analyses comparing different concentrations of each SF separately were generally significant ($P \leq 0.016$); exceptions were Cohort 1 *B. amphitrite* cyprids to *B. crenatus* and *B. improvisus* SFs and Cohort 2 *E. modestus* cyprids to *B. improvisus* SF. A Dunn's

multiple comparison was carried out to determine where significant differences occurred and results are indicated in Figure 6.4. Significantly higher settlement occurred at concentrations of 1, 10 and 100 $\mu$g ml$^{-1}$ for *B. amphitrite* cyprids and at 10 and 100 $\mu$g ml$^{-1}$ for *E. modestus*. For both species, significantly higher settlement occurred most frequently at 10 $\mu$g ml$^{-1}$. The 10 $\mu$g ml$^{-1}$ concentration was selected for the comparison of all SFs. Kruskal-Wallis statistics comparing different SFs indicated that *B. amphitrite* settlement was significantly different only for Cohort 2 at a concentration of 10 $\mu$g ml$^{-1}$ (P = 0.001), while both *E. modestus* cohorts had significantly different settlement (P= 0.005, Cohort 1; and 0.008, Cohort 2). A Dunn's multiple comparison was carried out to determine where significantly differences occurred and results are indicated in Figure 6.5. *B. amphitrite* settlement to all SFs, except that of *B. improvisus*, was significantly higher than the control. *E. modestus* settlement in response to each SF was significantly higher than the control, except for *B. improvisus* and *E. modestus* SF.

**Experiments SF2 and SF3 (Nitrocellulose assays)**

The level of *E. modestus* settlement was higher in Experiment SF3 than SF2, while for *B. amphitrite* it was similar. Lowest settlement occurred on the control spots in all assays with both species. For *E. modestus*, conspecific settlement was highest in all assays. However, in *B. amphitrite* assays of Experiment SF2, conspecific settlement was highest only in Assay 2 (with *C. montagui* and *S. balanoides* SFs). *B. amphitrite* settlement was highest on *B. improvisus* SF in Assay 1 and 3. In Experiment SF3, *B. amphitrite* conspecific settlement was higher in Assays 1 to 4, where 2 allospecific SFs were present with the conspecific. However, in Assay 5, with all selected SFs present, conspecific settlement was exceeded by that of *B. improvisus, C. montagui* and *S. balanoides* SFs. Kruskal-Wallis statistics indicated that the results of all the *E. modestus* assays of Experiment SF2 were not significantly different, while for *B. amphitrite* Assay 1 and 2 results were significantly different (P = 0.004, Assay 1; and 0.001, Assay 2), but not those of Assay 3. For Experiment SF3, *E. modestus* settlement in Assays 1, 3, 4 and 5 was significantly different (P = 0.002, Assay 1; 0.006, Assay 3; 0.002, Assay 4; and 0.002, Assay 5), but not significant for Assay 2. *B. amphitrite* results for Assays 1, 2 and 3 were significantly different (P = 0.005, Assay1; 0.010, Assay 2; 0.004, Assay 3), but

(a)




(b)

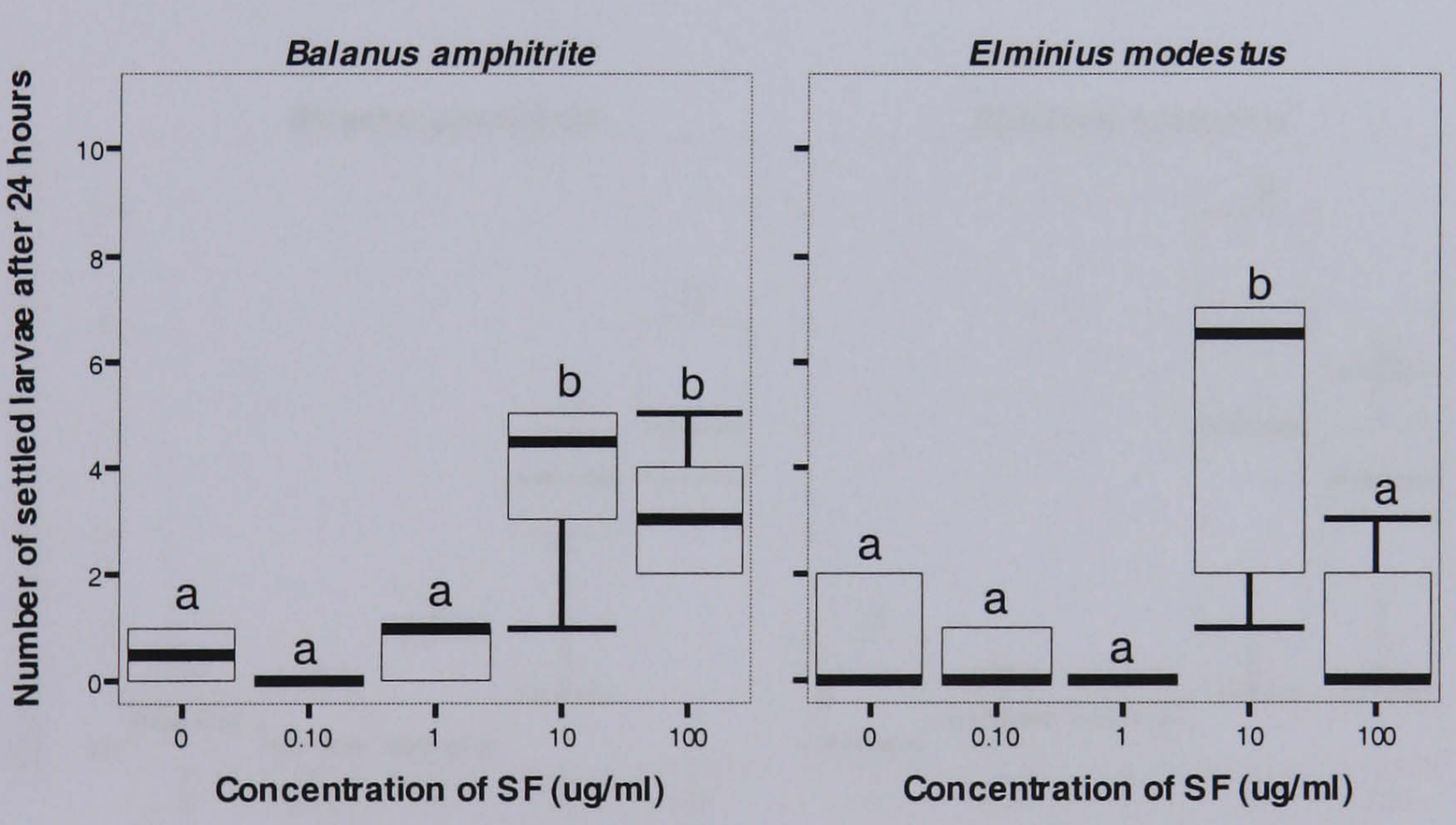
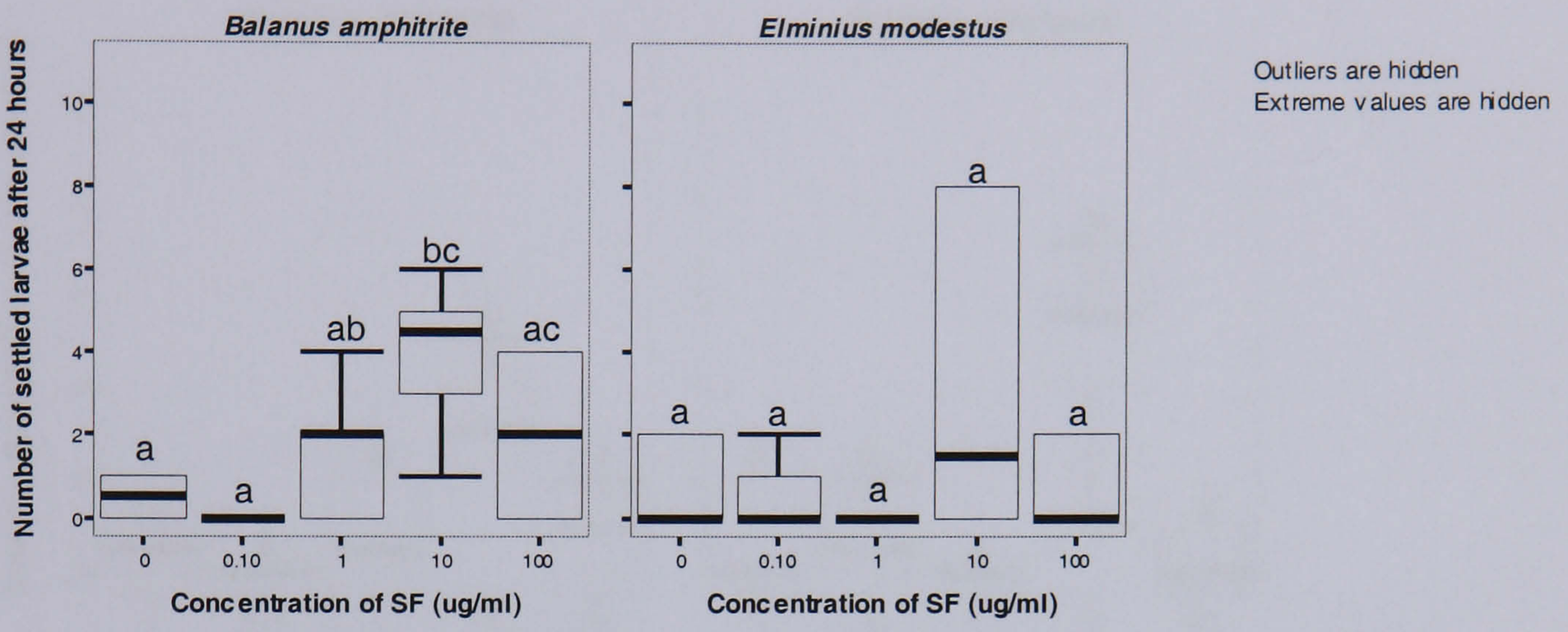



Figure 6.2: The effect of different concentrations of con- and allo-specific SF on 24-hour settlement by Day 1 *E. modestus* and *B. amphitrite* cyprids to con- and allo-specific SF. SF treatments are (a) *B. amphitrite*, (b) *B. crenatus*, (c) *B. improvisus*, (d) *C. montagui*, (e) *E. modestus*, (f) *S. balanoides*. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. Different letters above bars indicate significant differences, $P \leq 0.05$. N = 6. (Experiment SF1)

(c)




(d)

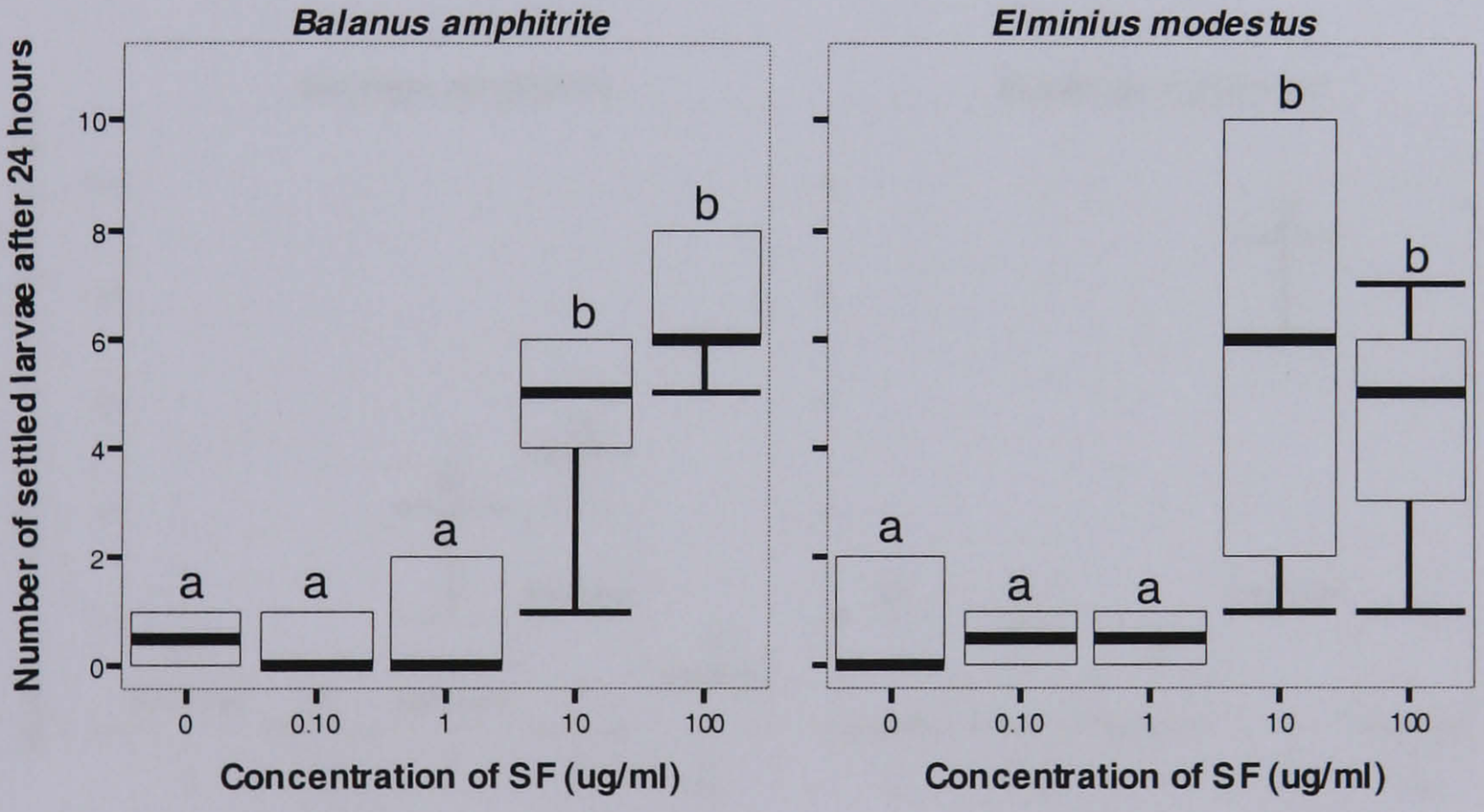
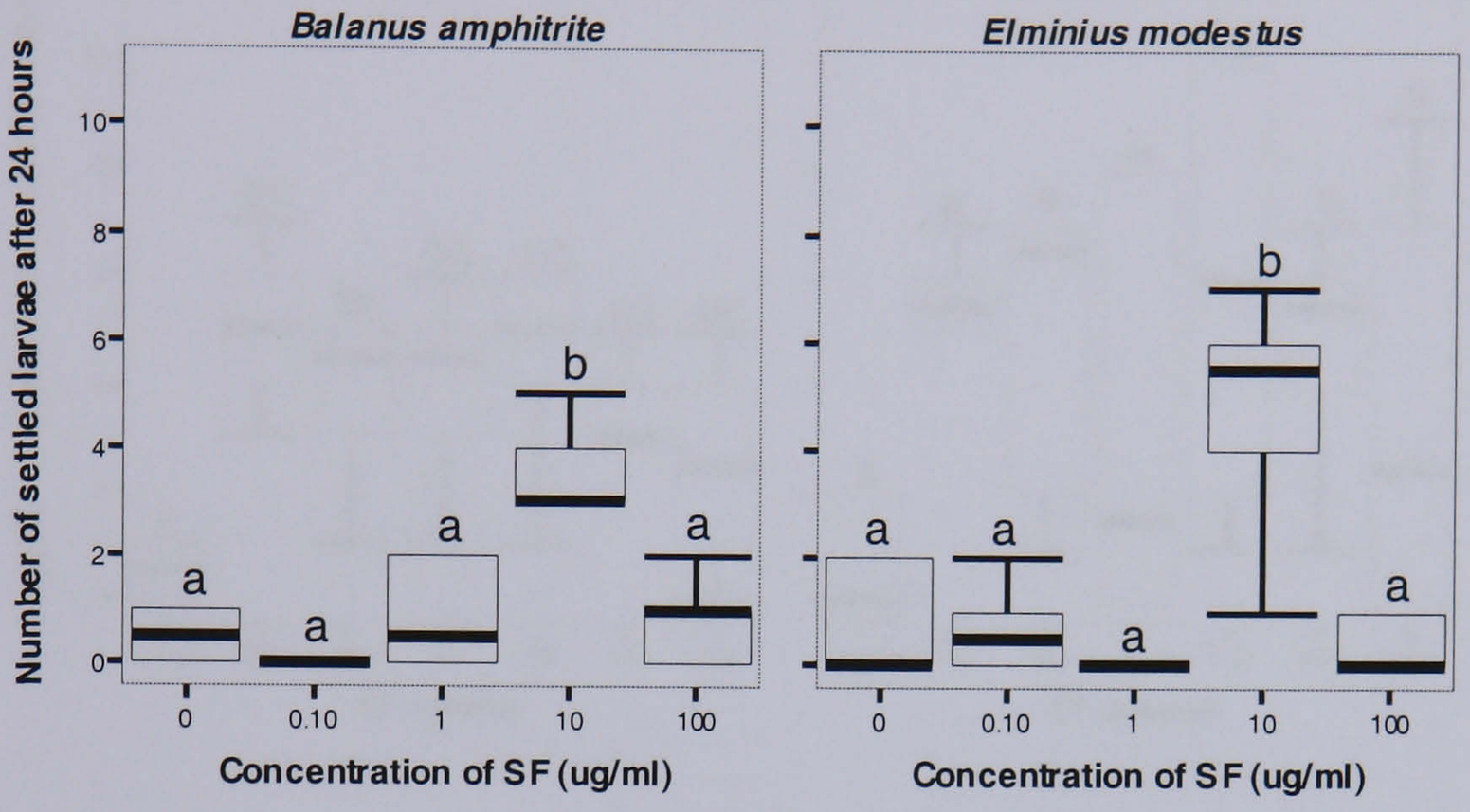



Figure 6.2 (continued): Con- and allo-specific settlement of Day 1 *E.modestus* and *B. amphitrite* cyprids. SF treatments are (c) *B. improvisus,* (d) *C. montagui*.

(e)




(f)

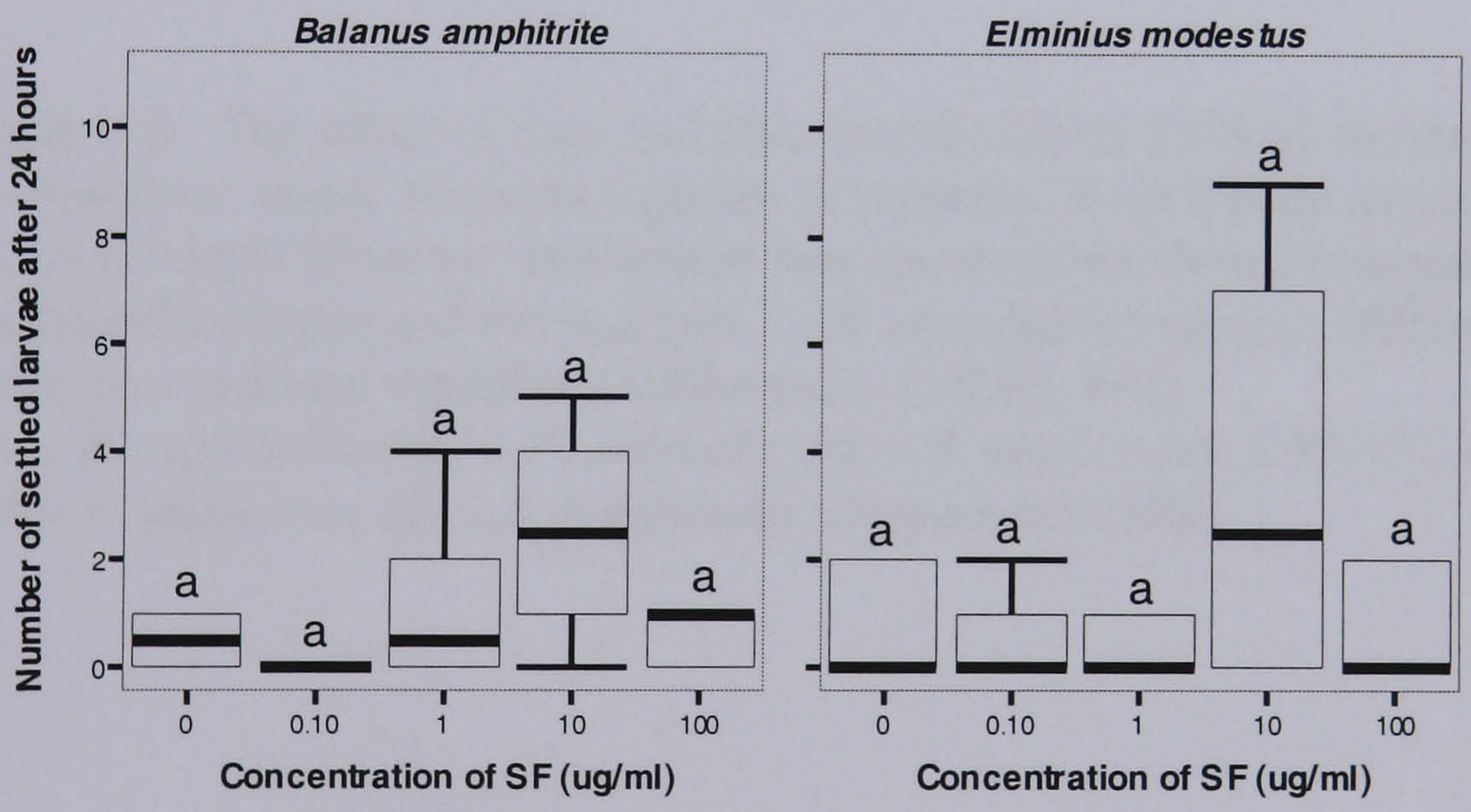



Figure 6.2 (continued): Con- and allo-specific settlement of Day 1 *E. modestus* and *B. amphitrite* cyprids. SF treatments are (e) *E. modestus* and (f) *S. balanoides*.

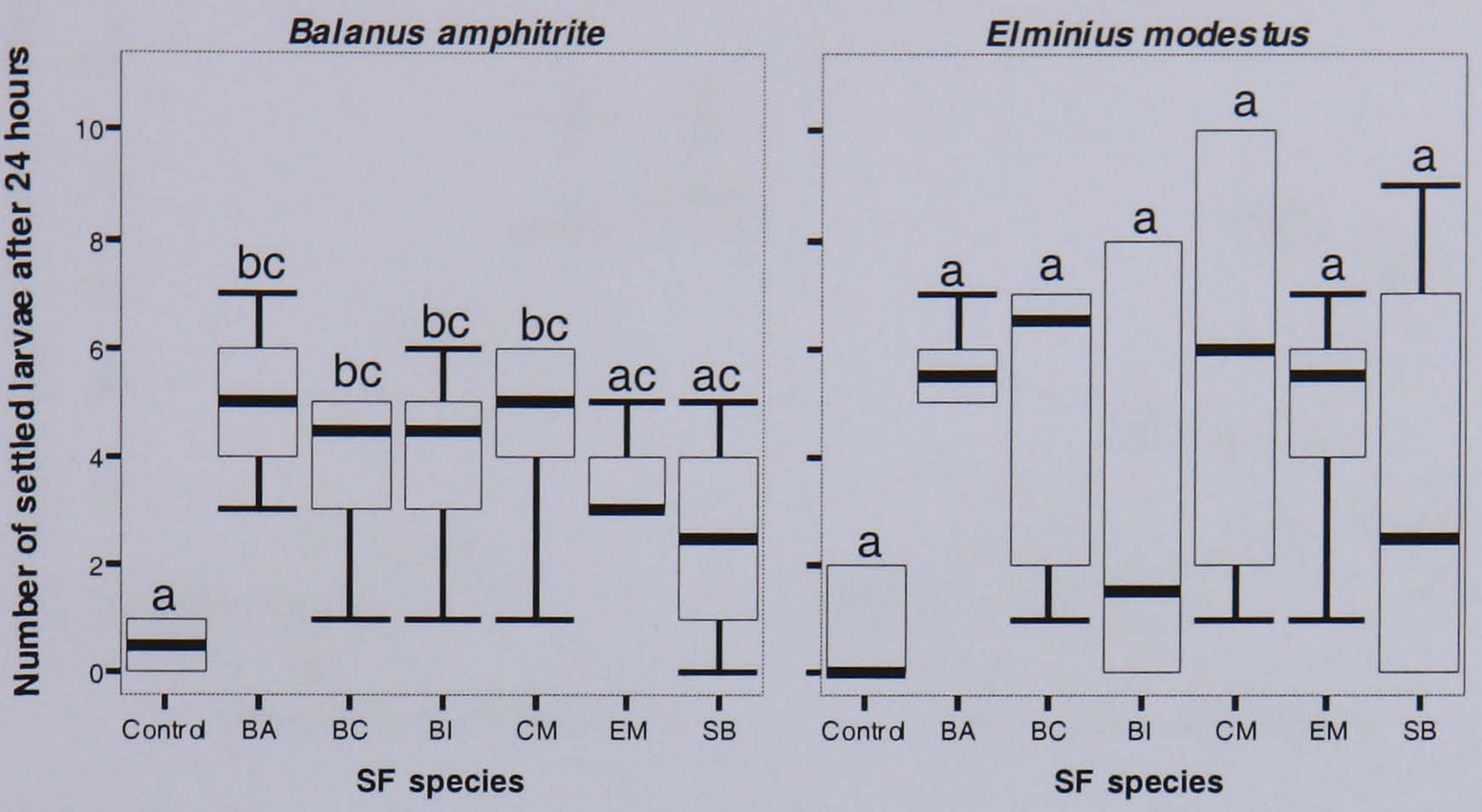
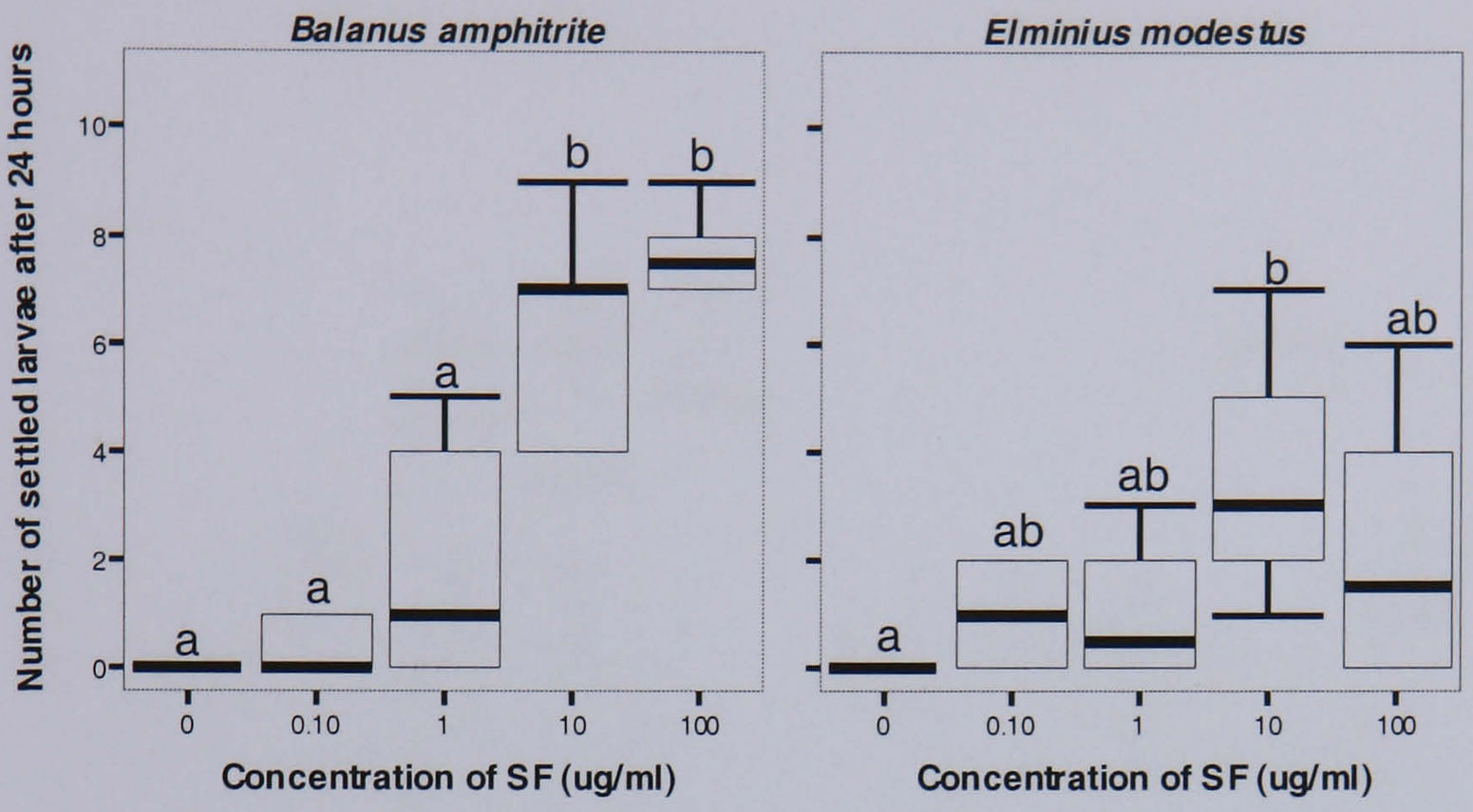



Figure 6.3: The effect of con- and allo-specific SF on 24-hour settlement by Day 1 *B. amphitrite* and *E. modestus* cyprids in separate 24-well plate assays with separate assays for each SF tested. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. Different letters above bars indicate significant differences, $P \leq 0.05$. N=6.
BA = *B. amphitrite*; BC = *B. crenatus*; BI = *B. improvisus*: CM = *C. montagui*: EM = *E. modestus*; SB = *S. balanoides.* (Experiment SF1)

(a)




(b)

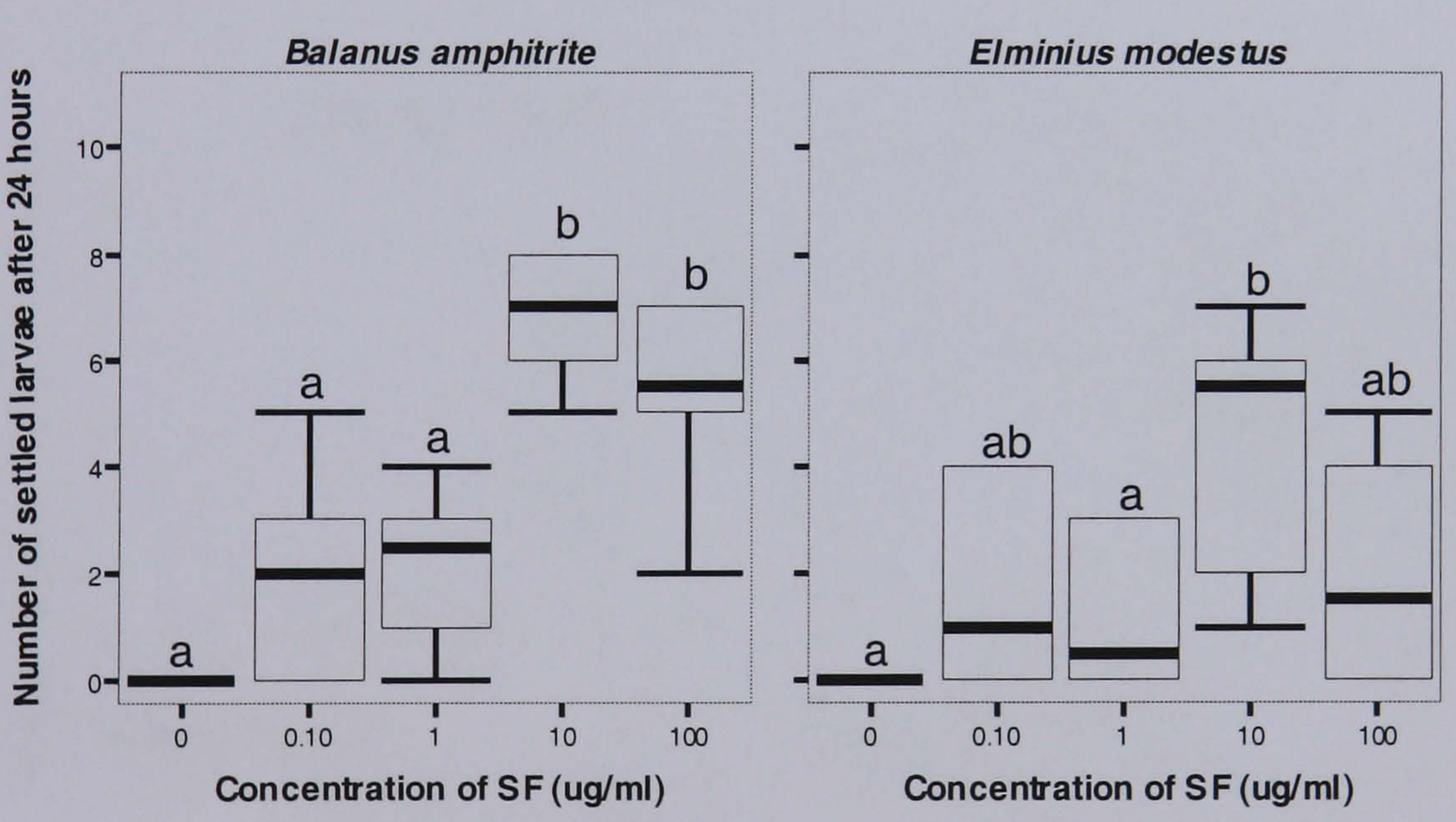
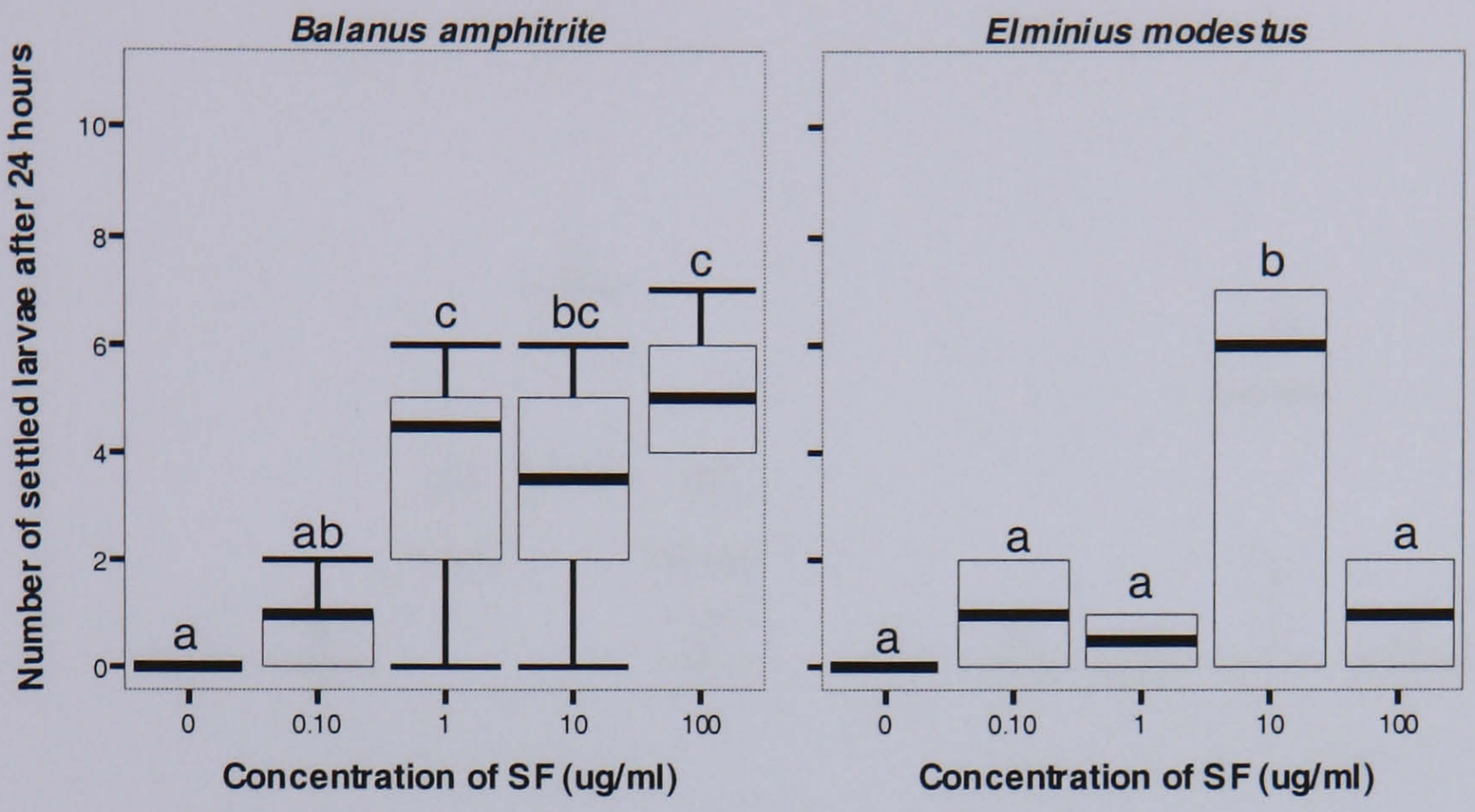



Figure 6.4: The effect of different concentrations of con- and allo-specific SF on 24-hour settlement by Day 4 *E. modestus* and *B. amphitrite* cyprids to con- and allo-specific SF. SF treatments are (a) *B. amphitrite*, (b) *B. crenatus*, (c) *B. improvisus*, (d) *C. montagui*, (e) *E. modestus*, (f) *S. balanoides*. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. Different letters above bars indicate significant differences, $P \leq 0.05$. N = 6. (Experiment SF1).

(c)




(d)

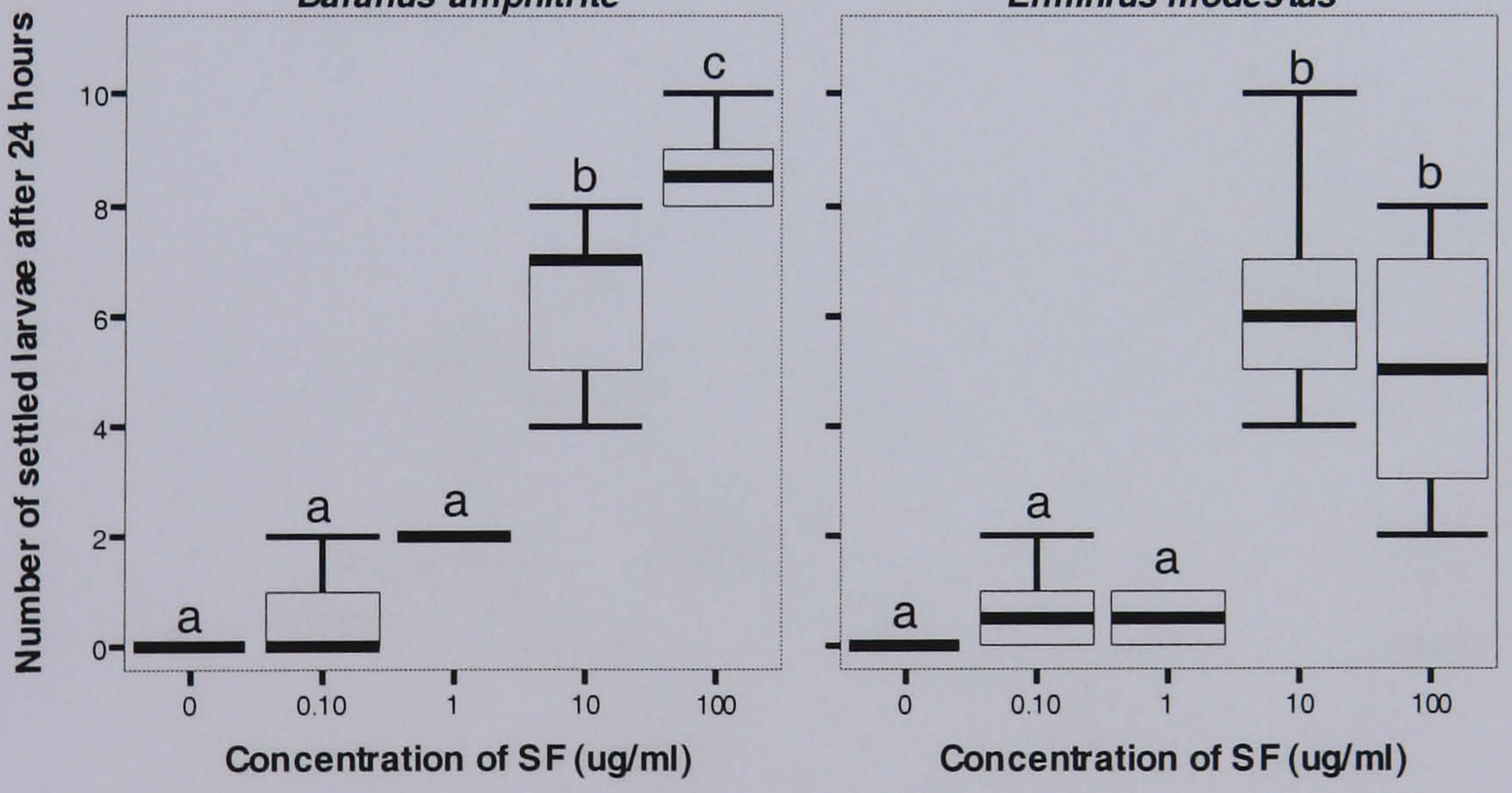
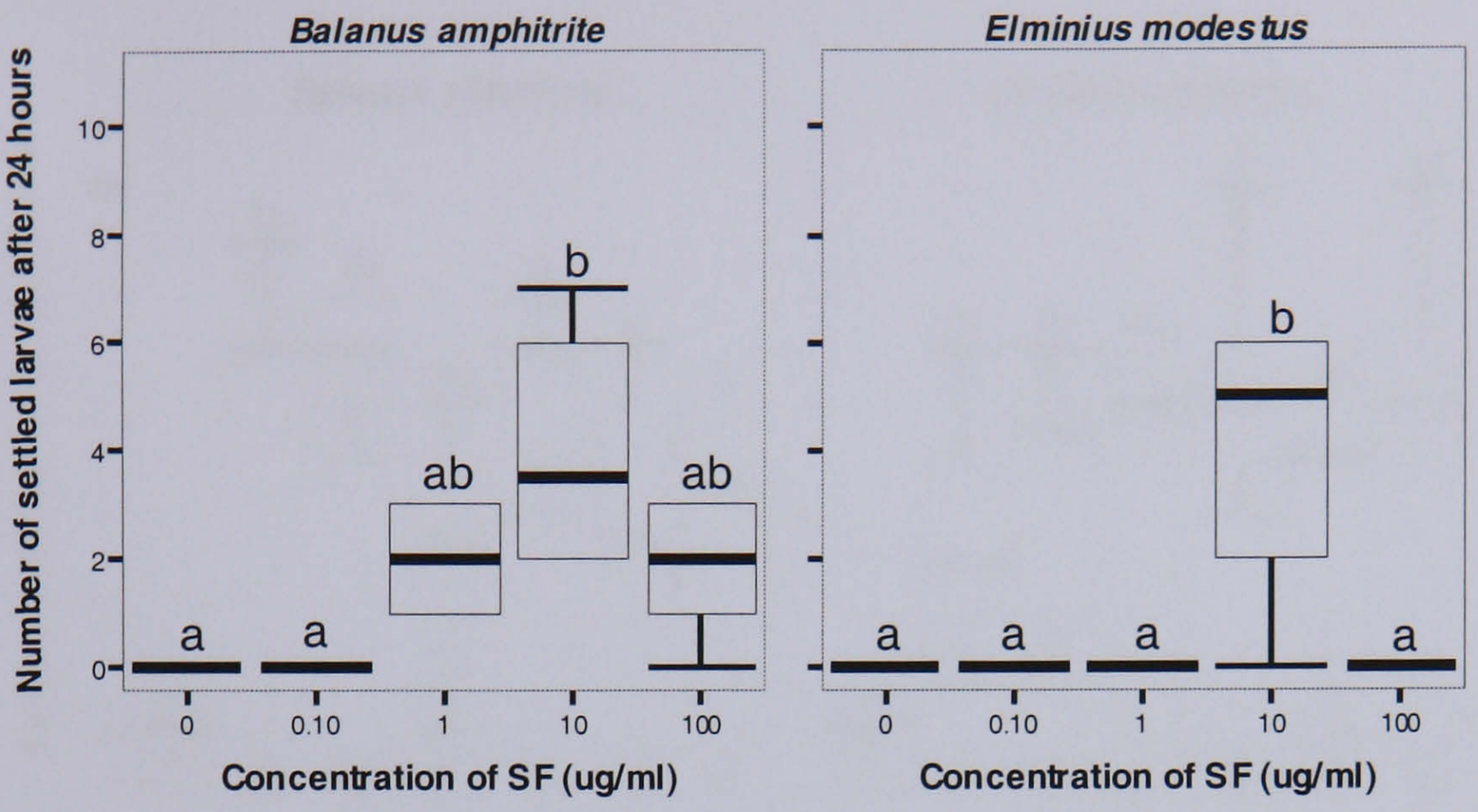



Figure 6.4 (continued): Con- and allo-specific settlement of Day 4 *E. modestus* and *B. amphitrite* cyprids. SF treatments are (c) *B. improvisus,* (d) *C. montagui*.

(e)




(f)

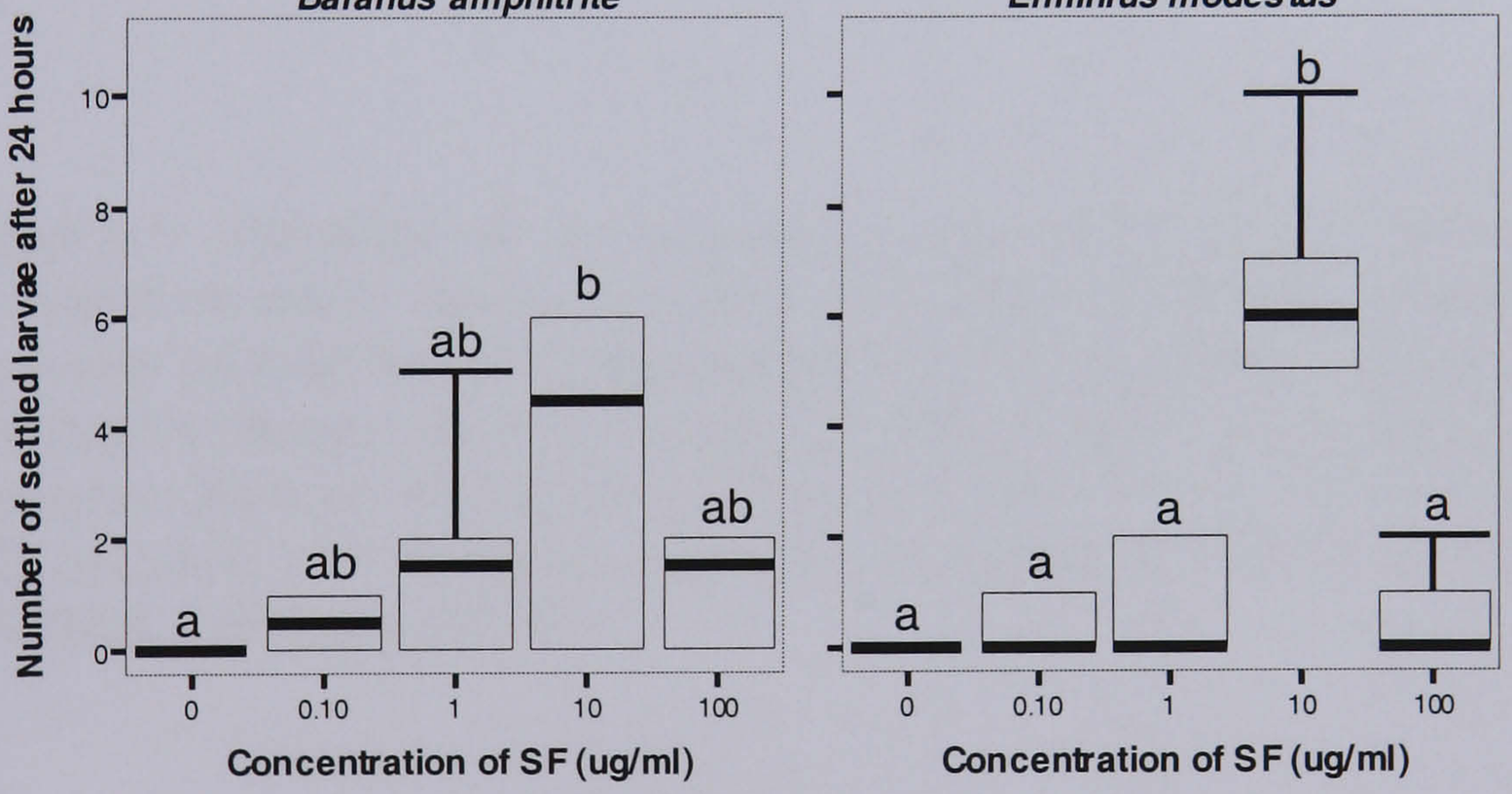



Figure 6.4 (continued): Con- and allo-specific settlement of Day 4 *E. modestus* and *B. amphitrite* cyprids in 24-well assays. SF treatments are (e) *E. modestus* and (f) *S. balanoides*.

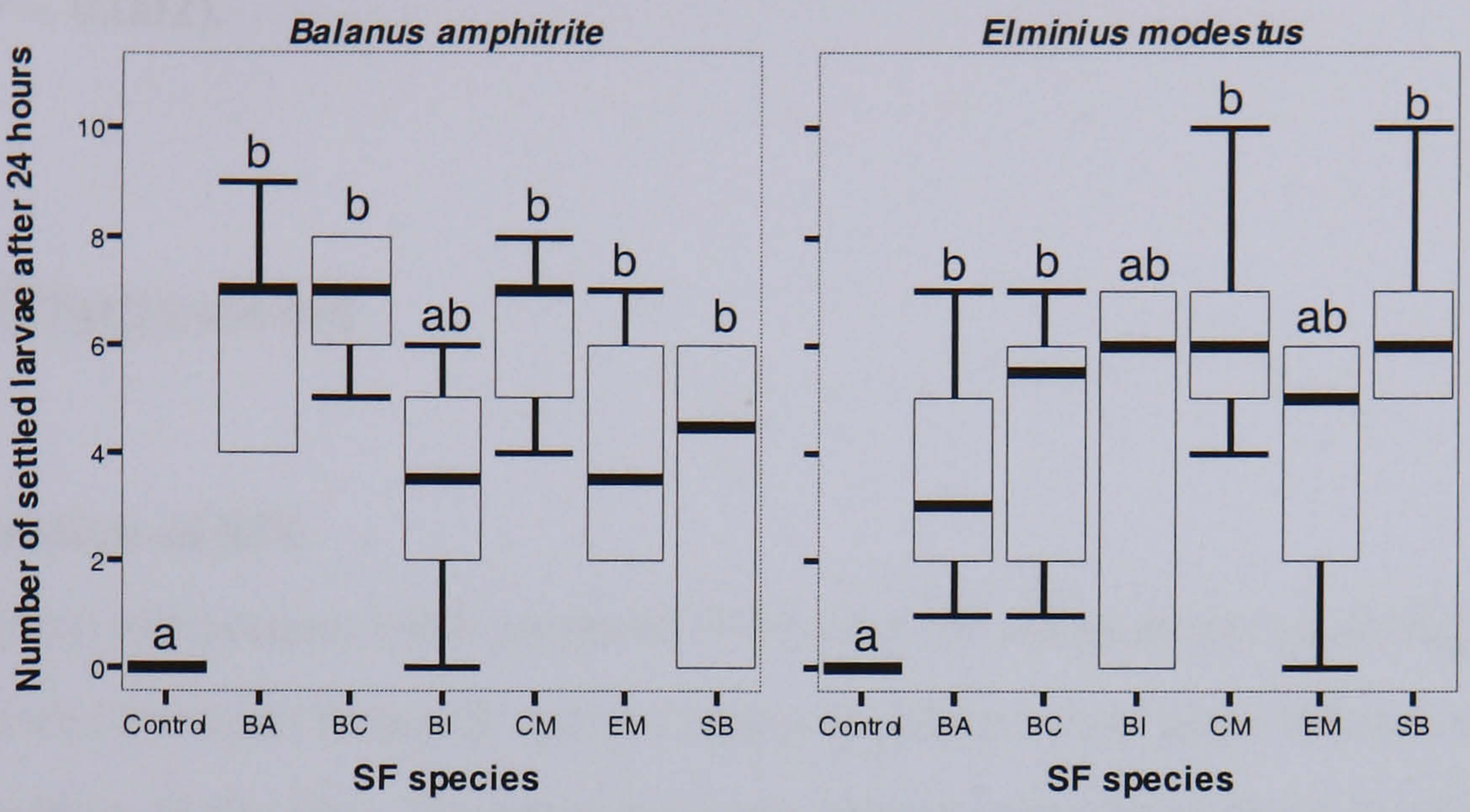



Figure 6.5: The effect of con- and allo-specific SF on 24-hour settlement by Day 4 *B. amphitrite* and *E. modestus* cyprids in separate 24-well plate assays with separate assays for each SF tested. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. Different letters above bars indicate significant differences, $P \leq 0.05$. N = 6.. BA = *B. amphitrite*; BC = *B. crenatus*; BI = *B. improvisus*: CM = *C. montagui*: EM = *E. modestus*; SB = *S. balanoides*. (Experiment SF1)

not significant for Assays 4 and 5. Overall, only one assay that included all selected SFs was significantly different; this was Assay 5 with *E. modestus* cyprids in Experiment SF3 ($P = 0.002$).

## 6.4 Discussion

### Preparation of SFs

All protein extractions were prepared following the same protocol, though inherent differences between barnacle species and populations may have influenced the composition of the SFs. Variation between extracts may have been mediated or exacerbated by different positive/negative effects of different sources of variation in each extract. Matsumura et al. (1998a) carried out experiments with a range of concentrations of crude extracts and purified SIPC. Settlement was dependent on the concentration used, with the level of settlement similar for 10 $\mu$g ml$^{-1}$ crude extract and 1 $\mu$g ml$^{-1}$ SIPC. Thus the settlement-inducing ability of SIPC was 10-fold that of crude extracts, and if SIPC was the sole inducer of settlement in the crude mix then the active protein may be ca. 10% of SF.

The higher protein content of *B. improvisus* was attributed to the use of freeze dried animals. This species aside, there was noticeable variation in total protein between other extracts when measured after the initial extraction. The concern was that the SIPC content may have been highly variable between different samples. However, the precipitation of protein by ammonium sulphate, undertaken primarily to concentrate the sample, produced a more uniform protein composition between samples. While all samples may have had a mix of similar proteins, the ratio of each may have been different in the first extract, but this would not preclude the presence of some proteins being at similar levels in all samples. Selective precipitation by ammonium sulphate may explain the uniformity of protein content in the concentrated extract. Globulin-like proteins, with substantial hydrophobic surface patches, are sensitive to salt concentration and their solubility decreases as salt content increases (Scopes, 1994). Thus, ammonium

sulphate precipitation of the protein mix would favour the precipitation of SIPC, an alpha-2-macroglobulin, though not exclusively so. The uniformity of protein content of the concentrated samples (except that of *B. improvisus*) may indicate that SIPC-content of the extracts was comparable. The variation in the intensity of the immunostaining of the same protein volume of each extract may be due to species specificity. The relatedness of the selected species may be expressed:-

(*B. amphitrite*: *B. crenatus*: *B. improvisus*) *S. balanoides*) *E. modestus*) *C. montagui*)

(Pérez-Losada et al., 2004).

Thus the strong signal from *B. improvisus*, followed by that of *B. crenatus*, concurs with this, although the *B. crenatus* signal was noticeably less intense than that of *B. improvisus* SF. The *E. modestus* signal appears contrary to the relatedness order. However, during protein purification (Chapter 5), it was realised that the staining results from 2 bands in close proximity. Thus, a single band may be within the suggested relatedness order. The reduced intensity of *S. balanoides*, and finally *C. montagui*, also matched the proposed relatedness. As intensity of staining was generally comparable to relatedness of species, this may also indicate SIPC content of the SFs was similar, though variation in SIPC content between SFs cannot be excluded. The antibody used was *B. amphitrite*-specific, and therefore would be expected to give the strongest signal with this species and the lower intensity of other samples may be indicative of sequence and structure variation of SIPC between species.

The SFs were not boiled during preparation. Crisp and Meadows (1962) reported that boiled barnacle extracts had a more intense settlement response than unboiled extracts, though more recently it has been shown that for *B. amphitrite* extract boiling reduced protein content and settlement activity by ca. 80% (Matsumura et al., 1998a). The prime reason for boiling was to remove bacteria that had an inhibitory effect on settlement (Crisp and Meadows, 1962). The final stage of the current preparation of extracts was to filter through a 0.2 $\mu$m filter, which removed bacteria, and bacterial contamination was not observed in assays.

**Selection of cyprid ages**

Day 1 and Day 4 cyprids of both species were selected for 24-well plate assays. Thus *E. modestus* assays were carried out with both 'decisive'/'early settlers' and 'desperate'/'late settlers' (Chapter 4), but at an age when discriminatory ability was still expected. The selection of Day 1, rather than Day 0 cyprids, allowed the same age to be selected for each species, given the low settlement of Day 0 *B. amphitrite* cyprids (Rittschof et al., 1984). Day 4 settlement was anticipated to be at an effective level of discrimination for both species. Nitrocellulose assays were carried out with Day 0 *E. modestus* and Day 1 *B. amphitrite* cyprids. Trial nitrocellulose assays indicated low settlement by *E. modestus* in this environment and it was considered important to carry out the assays using cyprids that were both discriminating and at their highest settlement ability.

**24-well plate assays**

The lack of discrimination by *E. modestus* cyprids between different species' SFs accords with the findings of Knight-Jones and Moyse (1961) and Larman and Gabbott (1975), but contradicts the findings of others (e.g. Barnett et al., 1979; Barnett and Crisp, 1979). The lack of discrimination by *B. amphitrite* cyprids accords with the findings of Kato-Yoshinaga et al. (2000), but is contrary to Matsumura et al. (2000). In both *B. amphitrite* examples, the nitrocellulose method was used and cyprids were able to 'choose' between species in a single assay, whereas, in this 24-well plate experiment, they were faced with an extract from a single species. Statistical analysis implied that if a cyprid of either species was faced with an adult cue from one species only, then an allospecific cue was likely to be as potent as the conspecific, and there was no evidence that age affected discriminatory abilities. A relationship between systematic affinities was not found.

The fact that peak *B. amphitrite* settlement occurred at either 10 or 100 $\mu$g ml$^{-1}$ dependent on SF may be an indication that SIPC content varied between the SFs. Statistical analysis indicated that SF concentration was important to settlement for both species. Crisp and Meadows (1962) reported that different threshold concentrations of

con- and allo-specific extracts were required to stimulate *S. balanoides* and *E. modestus* cyprids, and *B. amphitrite* behaviour may be similar. *E. modestus* cyprids of both ages were highly sensitive to the SF concentration with low settlement at all concentrations except 10 $\mu$g ml$^{-1}$ for all SFs tested. If *E. modestus* cyprids were responding to different quantities of SIPC in the different SFs, the threshold concentration of different species' SFs may have less variation for *E. modestus* cyprids than *B. amphitrite* cyprids. As the peak settlement concentration by *B. amphitrite* cyprids varied between cohorts, sensory ability between individuals of different cohorts may vary. This may be due to individual physiology, or may be attributed to larval fitness and/or cohort variation. Settlement by *B. amphitrite* cyprids occurred more across the range of concentrations for each SF than by *E. modestus*. The effect of the difference in behaviour in the natural environment can only be speculated. The variation may be indicative of variation in sensory ability between individuals, or an indication that *B. amphitrite* may be able to tolerate a greater range of conditions. Thus, settlement may be less likely to be delayed, which may give juveniles a competitive advantage in post-metamorphosis fitness.

**Nitrocellulose assays**

The nitrocellulose assay may better reflect the natural environment, as cyprids about to settle in the wild may encounter adults of several species. As settlement by both species was always lowest on the control spots, individuals were discriminating. Overall, statistical analysis implied that cyprids preferred to settle close to another barnacle, though an allospecific cue was likely to be as potent as the conspecific. The congregation of cyprids at different treatments may have been dynamic, as larval density on treated areas, resulting in intraspecific competition for space, may have encouraged cyprids to relocate to a less dense area. Settlement may have been due to the direct influence of the treatment on the chosen location, or by one or more extracts encountered during searching activity and settlement may be a result of a culmination of these interactions. Larva-larva interactions (Matsumura et al., 2000), as a result of congregating cyprids, may have been a secondary influence on settlement preferences and may have brought about settlement irrespective of the origin of the SF.

The purpose of the various combinations of two cues in individual assays was to determine preferences, as behaviour may change dependent on the species represented by the cues. Again, like the 24-well plate assays results, systematic affinity did not influence settlement preferences. Earlier nitrocellulose membrane assays with *B. amphitrite* (Matsumura et al., 2000) tested the conspecific cue and that of *S. balanoides* simultaneously in small 6-spot assays with all spots treated and results indicated that conspecific settlement was significantly higher than allospecific. However, in separate nitrocellulose assays with extracts from one of three species and a blank control (Kato-Yoshinaga et al., 2000), *B. amphitrite* settlement in response to the different species was not significantly different, which agrees with present findings. In the current experiment, the result suggests that cyprids do not discriminate between SFs. However, the use of 3 or more SFs in the confined 'closed' space of the assay container may have influenced cyprid behaviour, and suggests that this approach may be unsuitable for testing more than 2 cues in a single laboratory assay.

## 6.5 Conclusion

The final protein content expressed as gram$^{-1}$ of starting weight of barnacle were similar for all SFs, suggesting that retained proteins were in a similar quantity in all selected species. The immunostaining of the selected SF extracts indicated that SIPC-like proteins were present in all species selected. The *B. amphitrite* SF had the most intense staining, while the intensity of staining of other species concurred generally with the latest findings on species relatedness.

In all experiments, SF induced more settlement compared to the control and generally the difference was significant, providing evidence of the discriminatory ability of cyprids. The results of both the 24-well plate and the nitrocellulose membrane assays indicated that an allospecific cue was likely to be as potent as the conspecific in inducing settlement, and settlement preferences were not influenced by species relatedness. When cyprids were faced with a single cue in 24-well assays, all SFs

induced equal settlement. The results of the nitrocellulose 'choice' assays were inconclusive. The lack of discrimination between SFs may indicate that this method is unsuitable for testing multiple cues at one time.

# Chapter 7

# Field experiments on con- and allo-specific settlement behaviour

# 7.1 Introduction

The *S. balanoides* laboratory assay results presented in Chapter 4 were far from conclusive, as the species did not adapt well to the laboratory conditions. The opportunity remained to investigate settlement behaviour in the natural environment by adapting laboratory experiments to provide data that may be compared to laboratory findings on other species. Field experiments were undertaken in each of the three years of the research project; the first simply to trial methods and inform full-scale experiments in the second and third years. The selection of SFs mirrored the laboratory experiments (6.1), and the same species' SFs, excluding that of *B. amphitrite*, were tested. Field experiments are unable to control environmental parameters, such as temperature, light, larval numbers and hydrodynamics, all of which may influence delivery of larvae to surfaces and/or their behaviour. Conversely, the unnatural environment of the assay container may also be an unwanted influence on activity. Thus, it would be useful to compare laboratory and field results for a single species, and therefore a field trial was also carried out for the species *E. modestus* in the final year of the project. Figure 7.1 is a map of Great Britain with field experiment sites marked.

# 7.2 Materials and methods

## 7.2.1. *Semibalanus balanoides*

*S. balanoides* has an annual reproductive cycle with settlement of cypris larvae occurring during April-June each year with maximum settlement anticipated during May (Crisp, 1956; Todd, 2003). Accordingly, field experiments were performed during May of each year, 2002 to 2004, as described below :-


Figure 7.1: Map of the Great Britain illustrating the locations of the sites used for field experiments during the research project.

**Trial experiments during 2002**

A field trial was carried out at Seaham, Co. Durham (54°20.8'N 2°48.5'W) to test the utility of newly-designed experimental slate panels. It was anticipated that the results of these trials would inform the experimental design of full-scale experiments in subsequent years. (Experiments SBF1.1-SBF1.3).

**Experiments during 2003**

Experiments were planned for St Andrews Bay, Fife (56°20.8'N 2°48.5'W). However, the cyprids arrived earlier than anticipated and the experiments were performed after the main settlement period. Planned experiments were curtailed and alternative limited experiments were carried out (Experiments SBF2.1-SBF2.3).

**Full-scale experiments 2004**

Full-scale experiments, postponed from 2003, were carried out at Tentsmuir Sands, Fife (56°24.8'N 2°48.5'W) (Experiments SBF3.1-SBF3.4).

## Trial experiments 2002

**Experiment objectives**

A. To test the durability of newly-designed 24-well slate panels.
B. To test the suitability of the slates for con- and allo-specific settlement assays.
C. To investigate through trial experiments con- and allo-specific settlement of *S. balanoides* larvae using a laboratory adapted method.
D. To decide appropriate experiment parameters for full-scale field experiments to investigate con- and allo-specific settlement of *S. balanoides* cyprids.

**Experimental design**

**Site description**

The Featherbed Rocks, Seaham, Co. Durham (54°20.8'N 2°48.5'W) was selected for the 2002 experimental trial. Figure 7.2 is a map of the area with the location of the experiment site identified, and Figure 7.3 is a photograph of the shore taken from the adjacent cliff top. The shore is a fixed platform of relatively smooth Magnesian

limestone rock from the Permian period (Smith, 1970) that is situated adjacent to and north of the harbour walls. With a long shore drift from north to south and the projection of the harbour walls into the open sea, the site was most exposed on its northern edge and more sheltered to the southern edge. The shore, although elevated from the beach, is a relatively flat expanse of rocks with an elevation of ca. $\leq 2$ metres from low to high water. The majority of the rocks were situated in the mid shore zone, with little high shore evident. and were heavily encrusted with a monoculture of *S. balanoides* (pers.obs.). Two locations were selected for the experiment. These were situated ca. 3 metres apart towards the southern edge of the shore, where the rocks had a suitable horizontal area for the attachment of the slate panels.

**Experiment SBF1.1**

The experiment investigated conspecific settlement compared to a blank control using the 24-well slate assay (2.3.1). The experiment was set out on the shore on May 12$^{th}$ 2002, and was removed on May 15$^{th}$ 2002. Eight slates were attached directly to the rock with four slates at each of two locations. The slates were positioned in a 2 x 2 block as illustrated in Figure 7.4. A rubber gasket and soft foam sheet were placed between the rock surface and the slate to act as a shock absorber to wave action. The rocks were drilled and plugged and slates attached by two screws through fixing holes at either end of the slate. Figure 7.5 illustrates the experimental design. Each block of four slates had two slate types; the first with all wells untreated, and the second with 12 randomly-selected wells treated with conspecific extract. The similarly-treated slates were placed diagonally opposite each other. Settlement was observed in situ after 2 and 4 tidal cycles (ca. 24 hours and 48 hours). The slates were removed after 6 tidal cycles (ca. 72 hours), and settlement was observed in the laboratory

**Experiment SBF1.2**

Experiment 2 was a repeat of Experiment 1. The experiment was set out on the shore on May 16$^{th}$ 2002, and removed on May 19$^{th}$ 2002. The same two site locations were used with the four slates attached in the holes drilled and plugged for Experiment SBF1.1. The positions of the treated and untreated slates were alternated.

**Experiment SBF1.3**

The experiment investigated con- and allo-specific settlement using the 24-well slate assay (2.3.1). The experiment was set out on the shore on May 20th 2002, and removed on May 23rd 2002. Eight slates, in two 2 x 2 blocks, were attached as described for Experiment SBF1.1. Each block of four slates had two slate types; the first with 12 randomly-selected wells treated with conspecific SF and the remaining 12 with allospecific SF from *E. modestus*, and the second with 12 randomly-selected wells treated with conspecific SF at Location 1, and allospecific SF, at Location 2, with the remaining 12 wells blank. The similarly-treated slates were placed diagonally opposite each other. Observations of settlement were carried out as before at ca. 24, 48 and 72 hours.

For all of these experiments the greatest concern was to establish if the experimental method could be used successfully in full scale experiments. Statistical analyses of the trial experiments were not expected to be conclusive, but were carried out to identify any adverse effect of the slate design on results and the level of replication appropriate for full scale experiments. A Kolmogorov-Smirnov test was carried out to determine if the data were normally distributed. The results of the test, combined with the fact that replicates of different treatments were not balanced within the experiments, lead to the selection of non-parametric tests for data analysis. Mann-Whitney U tests were used to compare 24-hour settlement data and Table 7.1 gives details of the data selections that were compared.

(a)


(b)


Figure 7.2: Maps showing the location of the *S. balanoides* 2002 experiment site at Featherbed Rocks, Seaham, Co. Durham (54° 50.1'N 1° 20.3'E); (a) Area map and (b) location map.

Figure 7.3: Photograph of the Featherbed Rocks, Seaham, Co Durham taking from the cliff top above the shore, looking south with the harbour walls to the right of the picture.

Figure 7.4: Photograph illustrating the block of four slates in a 2 x 2 array attached directly to the rocks at Location 1.

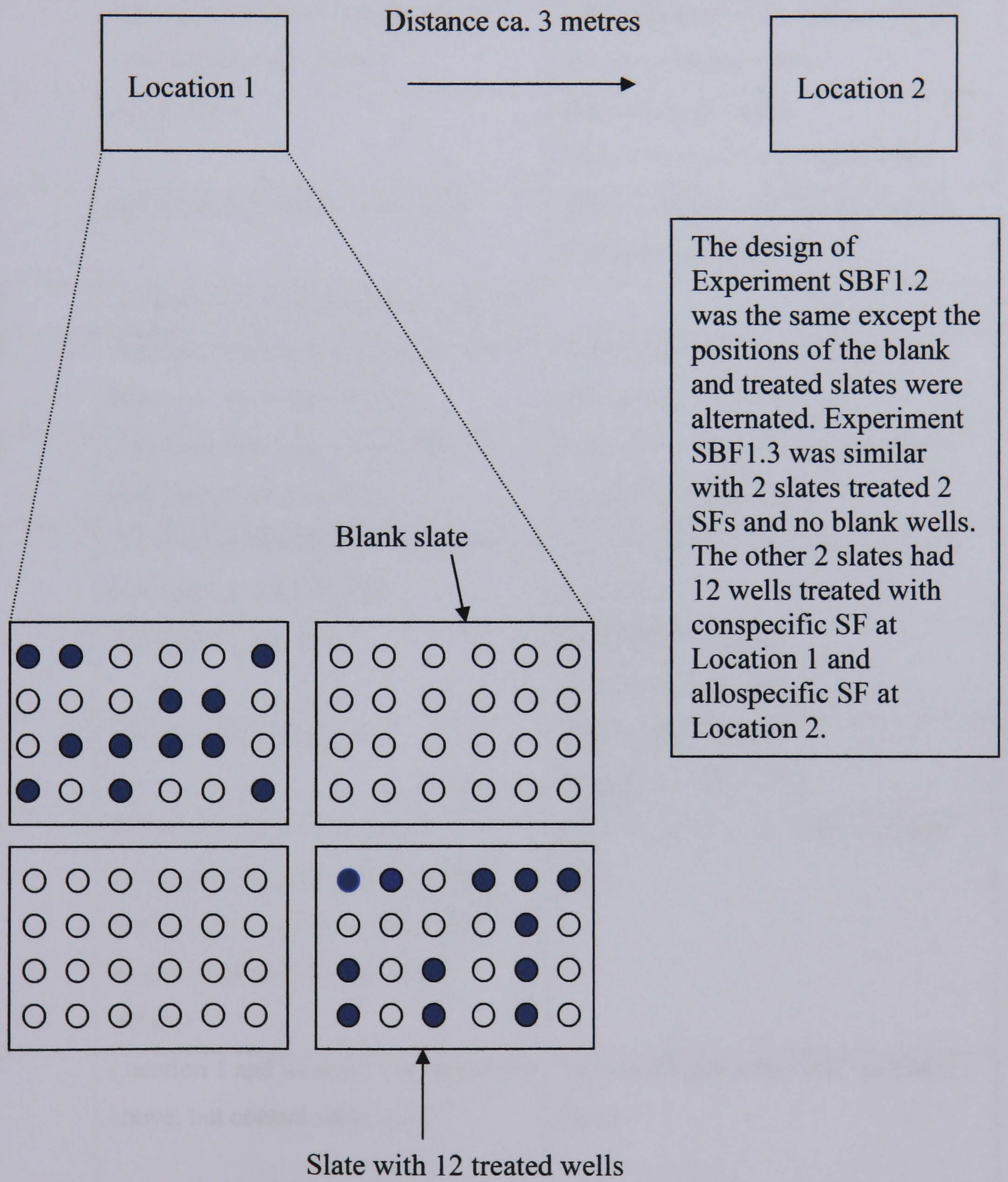



Figure 7.5: Diagram illustrating the layout of Experiment SBF1.1 of the field trial carried out to investigate *S. balanoides* larval settlement.

| Experiment | Selected slates | Comparison |
|---|---|---|
| Exp.1 | All slates with both conspecific SF and control wells (SB/C) | Individuals per well, conspecific SF to control treated wells |
| Exp.1 | All slates | Individuals per well, SB/C slates to Control only slates |
| Exp.1 | All slates, but control wells only | Individuals per well, SB/C slates to Control only slates |
| Exp.2 | As described for experiment 1 above | |
| Exp.3 | All slates with both conspecific and *E. modestus* SF wells (SB/EM) | Individuals per well, conspecific to allospecific SF treated wells |
| Exp.3 | All slates with both conspecific SF and control wells (SB/C) | Individuals per well, conspecific SF to control treated wells |
| Exp.3 | All slates with both *E. modestus* SF and control wells (EM/C) | Individuals per well, conspecific SF to control |
| Exp.3 | All slates at Location 1 | Individuals per well, EM/SB to SB/C slates |
| Exp.3 | All slates at Location 2 | Individuals per well, EM/SB to EM/C slates |
| Exp.3 | Location 1 slates with both conspecific SF and control wells (SB/C) and Location 2 slates with *E. modestus* SF and control wells (EM/C) | Individuals per well, SB/C to EM/C slates |
| Exp.3 | Location 1 and location 2 as described above, but control wells only | Individuals per well, SB/C to EM/C slates |

Table 7.1: List of statistical comparisons for *S. balanoides* field trial data (2002)

## Experiments 2003

### Experiment objectives

Due to the reduced larval supply at the time of the experiments (due to an unusually early settlement season), the planned experiment schedule was postponed with revised objectives developed ad hoc.

A. To investigate settlement behaviour of *S. balanoides* cyprids to con- and allo-specific cues using slate panels in a laboratory-adapted method.
B. To test the durability of the nitrocellulose assay in the natural environment.

### Experimental design

### Site description

The rocky shore at St Andrews Bay (56°20.8'N 2°48.5'W) was selected for the 2003 experiments. Figure 7.6 is a map of the area with the location of the experiment site identified, and Figure 7.7 is a photograph of the shore taken at sea level, facing north with the town of St Andrews visible on the skyline. The shore is sandstone with tight folds and faulting in the rock as a result of compressive earth movements during the Hercynian (McGregor, 1968). The shore is situated to the south of the bay in open sea that is within the outer reaches of the Tay estuary. Although the long shore drift is generally from north to south, the shore is subject to re-circulating currents such that the flow is reversed with the current approaching from the south (C.Todd pers.comm.). Many of the rocks were heavily encrusted with barnacles, in particular *S. balanoides* (pers.obs.).

Two locations were selected for the experiments situated within the central area of the mid shore. The experimental panels were laid horizontally, but the shore was prone to sand deposition, such that sand became trapped in the experimental wells of the slates. After initial assays, panels were relocated to vertical positions at two further locations ca. 5 m from the original sites. Figure 7.8 illustrates Location 1 (relocated) with experiment panels in the vertical position. Figure 7.9 is a diagram of the experiment

sites and panel positions. Location 2 was situated ca. 2 metres landward of Location 1. All panels faced the sea. At each location, Panel 1 is the panel to the left when facing it, and Panel 2 to the right. Additionally a neighbouring rock was selected for the trial nitrocellulose experiment. The panel was placed landward to reduce the wave action on this more delicate experimental medium.

**Experiment SBF2.1 (Multiple SF assays)**

The experiment was carried out from May 7th 2003 to May 11th 2003 using the 24-well slate assay (2.3.1). Two experimental panels, each with nine 24-well slates, were prepared and placed at Location 1 each day. Wells were treated in a latin square design with one of 5 SFs, namely *B. crenatus, B. improvisus, C. montagui, E. modestus* and *S. balanoides*, or were left blank (Figure 7.10). They were placed horizontally at Location 1 on May 7th and Location 2 on May 10th 2003. The panels remained on the shore for 4 tidal cycles (ca. 48 hours) with settlement observed at ca. 24 hours in situ using a hand lens and at ca. 48 hours in the laboratory using a binocular microscope.

**Experiment SBF2.2 (Multiple SF assays)**

The experiment period was carried out from May 13th 2003 to May 18th 2003 using the 24-well slate assay (2.3.1). Two panels, each with nine 24-well slates, were prepared and treated with SF as described for Experiment SBF2.1. They were placed at the relocated vertical positions as follows:-

| Date set out on shore | Location | Dates observed | |
|---|---|---|---|
| | | In situ | In lab |
| May 13th 2003 | Location 1 | May 14th | May 15th |
| May 14th 2003 | Location 2 | May 15th | May 16th |
| May 15th 2003 | Location 1 | May 16th | May 17th |
| May 16th 2003 | Location 2 | May 17th | May 18th |

All other parameters were as described for Experiment SBF2.1.

(a)


(b)


Figure 7.6: Maps showing the location of the *S. balanoides* 2003 experiment site at St Andrews Bay (56° 20.8'N 2° 48.5'W); (a) Area map and (b) Location map.

Figure 7.7: Photograph of the rocky shore at St Andrews Bay taken on the shore facing north with the town of St Andrews visible on the skyline.

Figure 7.8: Photograph of experiment panels in situ at Location 1 St Andrews Bay.

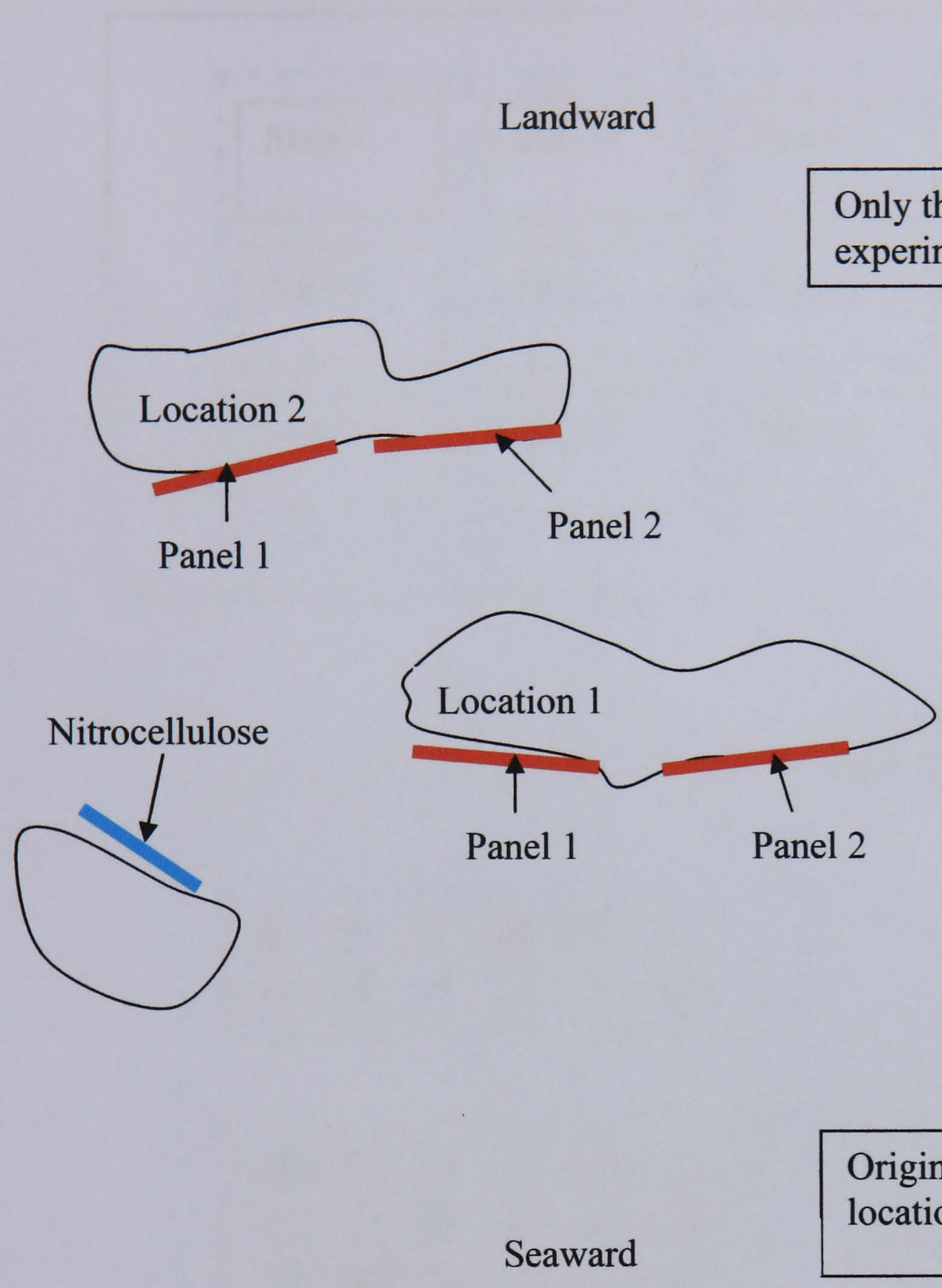



Figure 7.9 Diagram illustrating the relocated experiment site locations for field experiments at St Andrews Bay during 2003.

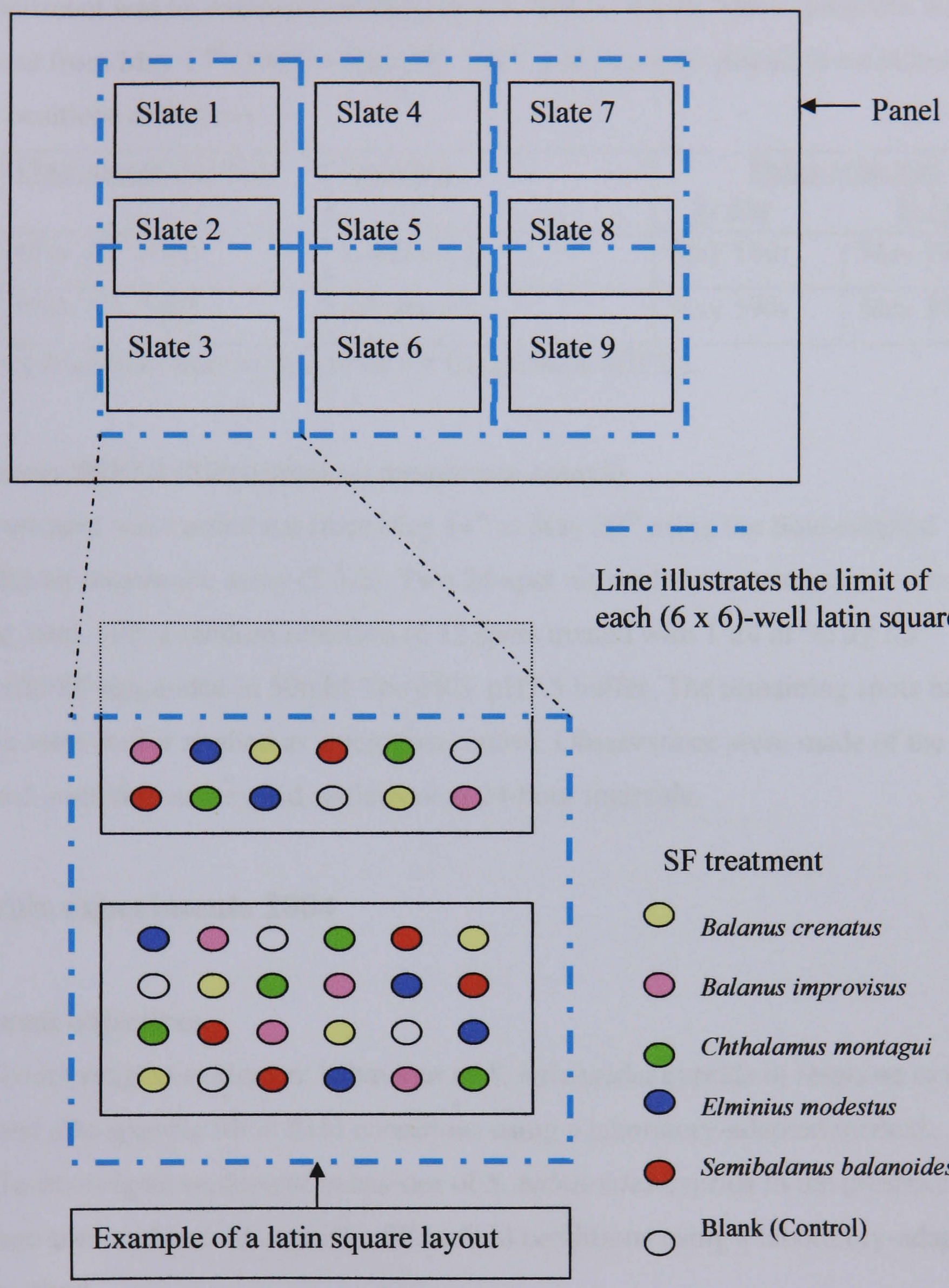



Figure 7.10: Diagram illustrating the panel layout and well treatments of Experiment SBF2.1

**Experiment SBF2.3 (Multiple SF assays)**

The experiment was an extension of Experiment SBF2.2 above. The experiment was carried out from May 17th 2003 to May 20th 2003, with 2 panels placed at the relocated vertical positions as follows:-

| Date set out on shore | Location | Dates observed | |
|---|---|---|---|
| | | In situ | In lab |
| May 17th 2003 | Location 1 | May 18th | May 19th |
| May 18th 2003 | Location 2 | May 19th | May 20th |

All other parameters were as described for Experiment SBF2.1.

**Experiment SBF2.4 (Nitrocellulose membrane assays)**

The experiment was carried out from May 14th to May 20th using the field-adapted nitrocellulose membrane assay (2.3.2). Two 24-spot nitrocellulose membranes were prepared, each with a random selection of 12 spots treated with 1 ml of 50 $\mu$g $ml^{-1}$ conspecific SF suspended in 50mM Tris-HCl, pH7.5 buffer. The remaining spots had 1 ml of the same buffer applied as a negative control. Observations were made of the membrane condition and cyprid settlement at 24-hour intervals.

## Full-scale experiments 2004

### Experiment objectives

A. To investigate settlement behaviour of *S. balanoides* cyprids in response to con- and allo-specific SF in field conditions using a laboratory-adapted method.
B. To investigate settlement behaviour of *S. balanoides* cyprids in the presence of one con- and one allo-specific SF in field conditions using a laboratory-adapted method.
C. To investigate settlement behaviour of *S. balanoides* cyprids to aged (biofilmed) slates in the presence and absence of a conspecific SF in field conditions using a laboratory-adapted method.
D. To investigate settlement behaviour of *S. balanoides* cyprids to conspecific SF using the field-adapted nitrocellulose membrane assay.

## Experimental design

### Site description

It was originally anticipated that the 2004 experiments would be carried out at St Andrews Bay, the location of the 2003 experiments. However, the experiments were carried out at Tentsmuir Sands, Fife (56°24.8'N 2°48.5'W) to reduce the risk of vandalism. Figure 7.11 is a map of the area with the site location identified and Figure 7.12 is a photograph of the shore taken from mean high water. The shore is an 8 km expanse of sand situated between the Tay estuary, 3 km to the north, and the smaller Eden estuary, 5 km to the south. At low tide an extensive sandbar is situated ca. 300 m seaward. It is an unpopulated coastline with Tentsmuir forest situated immediately inland of the shore. A small fresh water tributary flows into the sea ca. 10 m to the south of the site. Although the long shore drift is generally from north to south, the shore is subject to recirculating currents such that the flow is reversed with the current approaching from the south (C.Todd pers. comm.).

A line of World War II tank traps situated on the beach was selected as the experimental site. The concrete blocks were located within the mid shore range and were heavily encrusted with barnacles, in particular *S. balanoides*. *M. edulis* were also present, as well as some macroalgal species. While the relatively cubic shape of the blocks aided attachment of ropes and panels, many of them had irregularities, which limited suitable sites. Two blocks, ca. 3 m apart, were selected. Experimental panels were positioned vertically to reduce sand deposition in wells. Figure 7.13 is a diagram of the test sites and panel positions. Location 1 was the most northerly site and Panel 1 was attached to landward side with Panel 2 to the north side. At Location 2, Panel 1 was attached to the north-facing side, while Panel 2 was attached to the seaward side. Figure 7.14 is a photograph of Location 2 with panels in position. Additionally, nitrocellulose membrane trial experiments were carried out at on the landward side of Location 1 and seaward side of Location 2.

**Experiment SBF3.1 (Multiple SF assays)**

The experiment was carried out between April 28th and May 28th 2004 with experimental panels placed on the shore each day from April 28th to May 2nd 2004 (early settlement season), May 7th to May 11th 2004 (mid settlement season) and May 22nd to May 27th 2004 (late settlement season). The 24-well slate assay (2.3.1) was used with two panels, each with 9 slates, prepared and treated with SF as described for Experiment SBF2.1. Novel random designs were used throughout the experiment. The 24-hour settlement was observed in the laboratory using a binocular microscope.

**Experiment SBF3.2 (Two SF assays)**

The experiment was carried out over 3 days from May 3rd to May 5th using the 24-well slate assay (2.3.1). Both experiment locations were used (Figure 7.13) with 4 panels, each with 9 slates, prepared for each day. The wells of each of the 9 slates were treated randomly with two SFs, the conspecific and one allospecific, and a blank control, with 8 replicates of each treatment on each slate. The panels were located as follows:-

| Date | Location 1 | | Location 2 | |
|---|---|---|---|---|
| | Panel 1 | Panel 2 | Panel 1 | Panel 2 |
| May 3rd | SB/EM/Control | SB/EM/Control | SB/CM/Control | SB/CM/Control |
| May 4th | SB/BC/Control | SB/BC/Control | SB/BI/Control | SB/BI/Control |
| (SB=*S. balanoides*; BC=*B. crenatus*; BI=*B. improvisus*; CM=*C. montagui*; EM=*E. modestus*.) | | | | |

The 24-hour settlement was observed in the laboratory using a binocular microscope.

**Experiment SBF3.3 (Aged slate assays)**

The experiment was carried out between April 27th and May 12th using the 24-well slate assay (2.3.1). Experiment panels were placed on the shore on April 29th, May 2nd, May 7th and May 11th 2004. Two 9-slate panels were prepared for each of the four dates, and Location 2 was used for the experiment. Figure 7.15 illustrates the slate layout of each panel for the experiment. For each panel, 3 slates were aged in seawater (to develop a biofilm) for 48 hours at 12°C, 3 were aged similarly for 24 hours, while the remaining 3 slates were clean. The different slate types were attached to each panel in a latin square design. Random selections of 12 wells on each slate were treated with conspecific SF. The 24-hour settlement was observed in the laboratory using a binocular microscope.

(a)


(b)


Figure 7.11: Maps showing the location of the *S. balanoides* 2004 experiment site at Tentsmuir Sands (56° 24.8'N 2° 48.5'W); (a) Area map and (b) Location map.

Figure 7.12: Photograph of Tentsmuir Sands with World War II tank traps situated across the shore. The coastline to the north of the Tay estuary is visible on the horizon. The water in the foreground is fresh water from a small stream flowing directly to the sea.

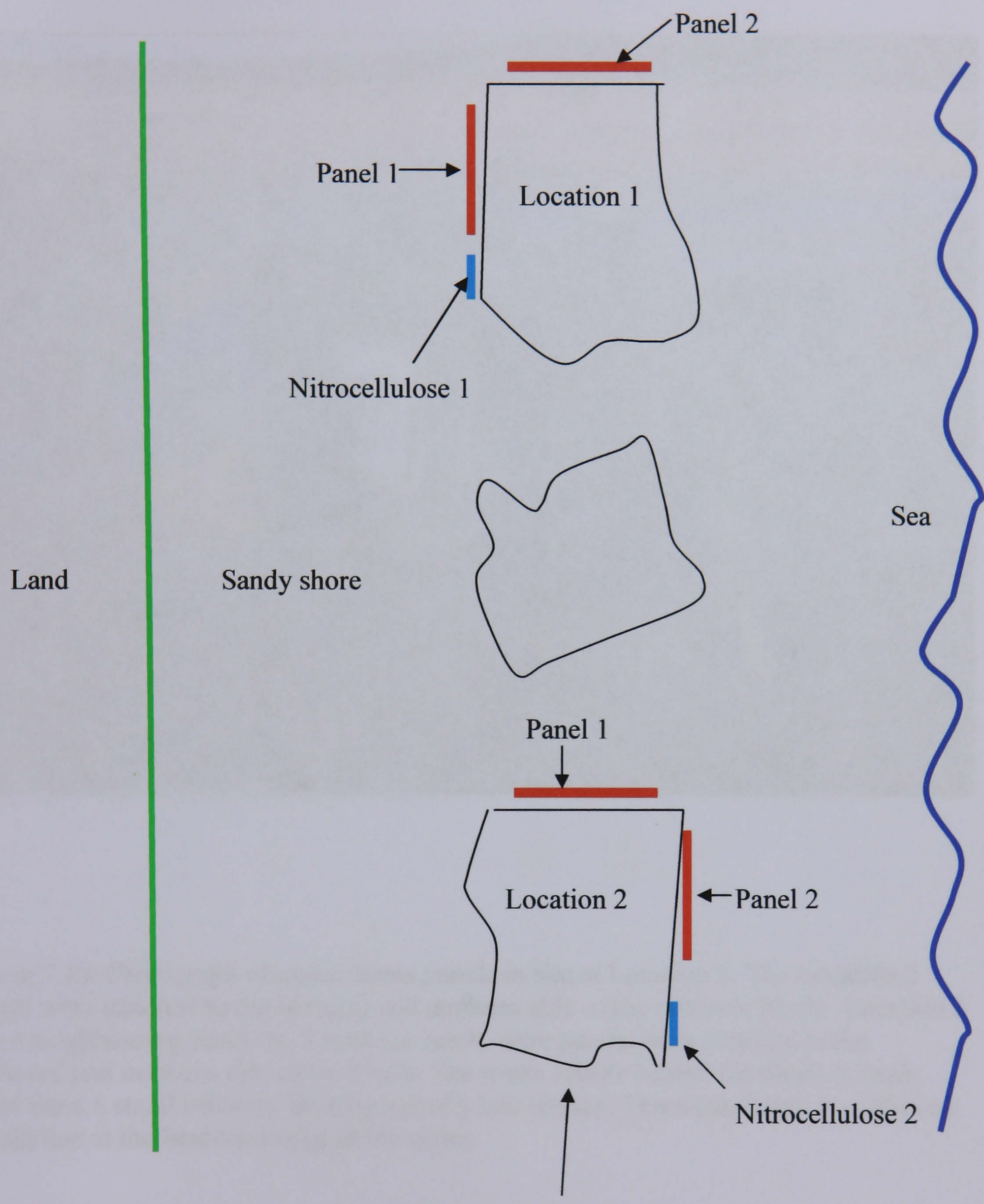



Figure 7.13: Diagram illustrating the field experiment locations at Tentsmuir Sands during 2004.

Figure 7.14: Photograph of experiments panels in situ at Location 2. The Location 2 panels were attached to the seaward and northern side of the concrete block. Location 1 was a neighbouring block ca. 3 m to the north, were panels were attached to the landward and northern side of the block. The water visible behind the block is fresh water from a small tributary flowing directly into the sea. Tentsmuir forest is visible on the skyline at the landward edge of the shore.

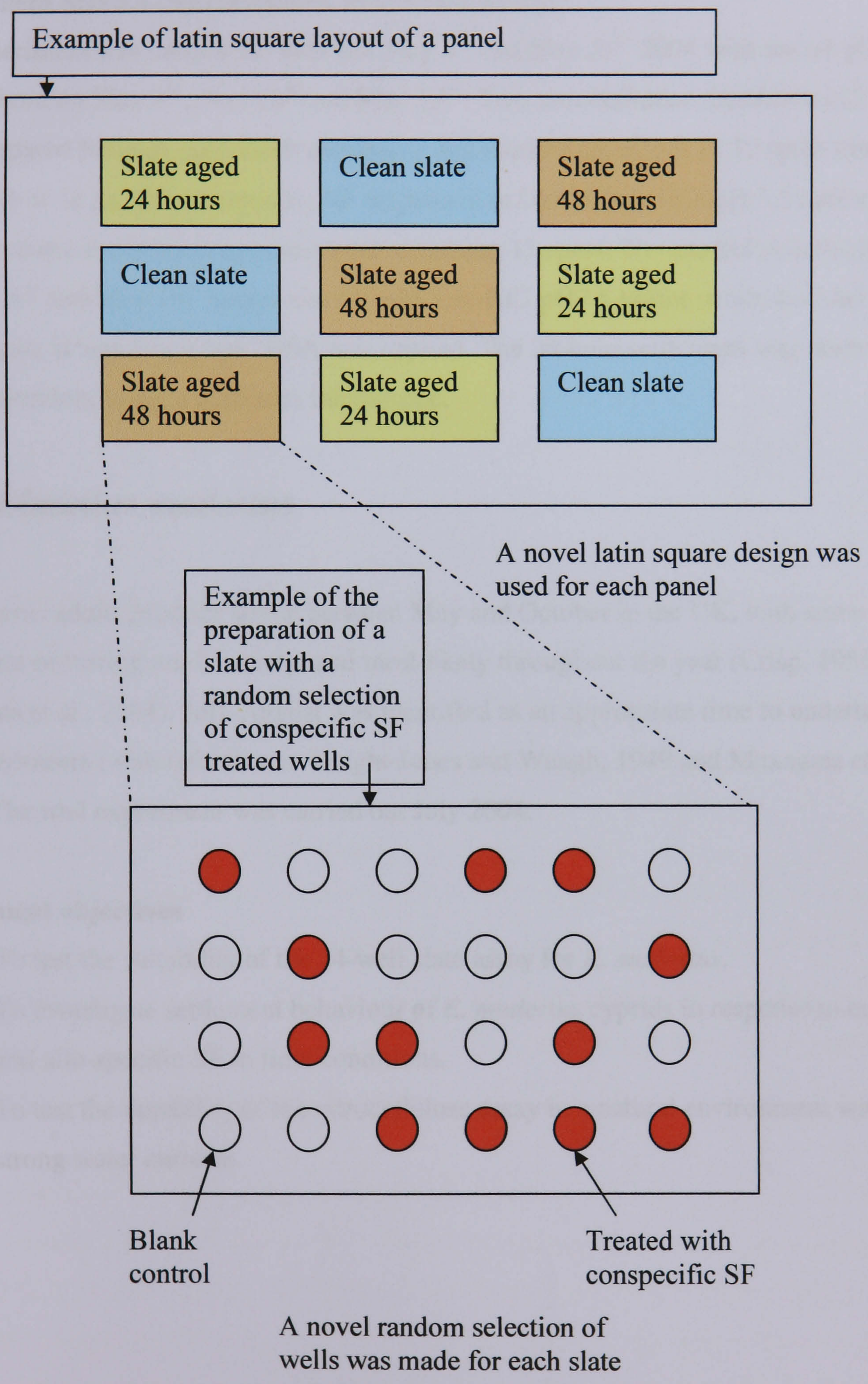



Figure 7.15: Diagram illustrating an example of the preparation of a panel for Experiment SBF3.2

**Experiment SBF3.4 (Nitrocellulose membrane assays)**

The experiment was carried out between May 8th and May 23rd 2004 with assays placed on the shore on May 8th, May 10th and May 22nd. Two nitrocellulose membranes (2.3.2) were prepared for each date. Each membrane had random selections of 12 spots treated with 1 ml of 50 $\mu$g ml$^{-1}$ conspecific SF suspended in 50mM Tris-HCl pH 7.5 buffer with 1 ml of a control treatment applied to the remaining 12 spots. The control selected for the May 8th and May 10th assays was 50 mM Tris-HCl pH7.5 buffer, while for May 23rd membranes, it was 50 $\mu$g ml$^{-1}$ BSA was applied. The 24-hour settlement was observed in the laboratory using a binocular microscope.

## 7.2.2 *Elminius modestus*

*E. modestus* adults produce larvae between May and October in the UK, with some settlement occurring until January, and most likely throughout the year (Crisp, 1958; Muxagata et al., 2004). July-August was identified as an appropriate time to undertake the experiments (with reference to Knight-Jones and Waugh, 1949 and Muxagata et al., 2004). The trial experiment was carried out July 2004.

**Experiment objectives**

A. To test the suitability of the 24-well slate assay for *E. modestus*.
B. To investigate settlement behaviour of *E. modestus* cyprids in response to con- and allo-specific SF in field conditions.
C. To test the durability of the nitrocellulose assay in a natural environment with strong water currents

## Experimental design

### Site description

Great Bull Hill, an area of the River Exe estuary at Exmouth, (50°36.9'N 3°25.8'W) was selected for the experimental trial. Figure 7.16 is a map of the area with the experimental site marked and Figure 7.17 is a photograph of the area taken from the edge of Great Bull Hill facing west. The site was accessible from Exmouth by walking across the estuary, a route that necessitated the crossing of the River Exe, such that the site could only be reached for ca. 5-7 days at low water spring tides. The site was covered by an extensive mussel bed with a virtual monoculture of *E. modestus* attached to the shell valves. The mussel bed was undulating with salt water pools remaining in the dips at low tide. A flat area on the top of a rise towards the centre of the bed was selected for the experimental site. Figure 7.18 is a photograph of the site. Two concrete-filled drainpipe frames were anchored into the mussel bed by roping them to metal L-shaped stakes. The experimental panels, each with nine slates, were attached to the frames using cable ties. A separate panel bearing the nitrocellulose membranes was attached similarly between the two frames.

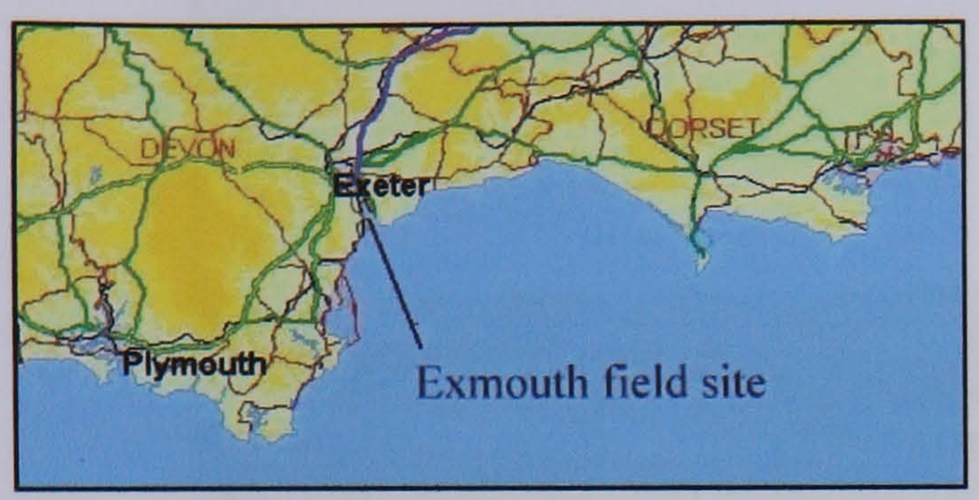
### Experiment EMF1 (Multiple SF assays)

The experiment was carried out over 6 days between July 2$^{nd}$ and July 7$^{th}$ 2004 with experimental panels placed on the shore on each day from July 2$^{nd}$ to July 6$^{th}$ 2004. The 24-well slate assay (2.3.1) was used. The panels were prepared and treated with SF as described for Experiment SBF2.1 (Figure 7.10). The 24-hour settlement was observed in the laboratory using a binocular microscope.

### Experiment EMF2 (Nitrocellulose membrane assay)

The experiment was carried out July 4$^{th}$ 2004. Two nitrocellulose membranes were prepared (2.3.2). Each membrane had random selections of 12 spots treated with 1 ml of 50 $\mu$g ml$^{-1}$ conspecific SF suspended in 50mM Tris-HCl pH7.5 buffer with a control treatment of 1 ml of the same buffer applied to the remaining 12 spots. The condition of the membrane and settlement was observed after 24 hours.

(a)




(b)


1 km

Figure 7.16: Map showing the location of the *E. modestus* 2004 experimental site at Great Bull Hill, Exmouth, Devon (50° 36.9'N 3° 25.8'W); (a) Area map and (b) Location map.

Figure 7.17: Photograph of Great Bull Hill Exmouth taken from the edge of the mussel bed facing west.

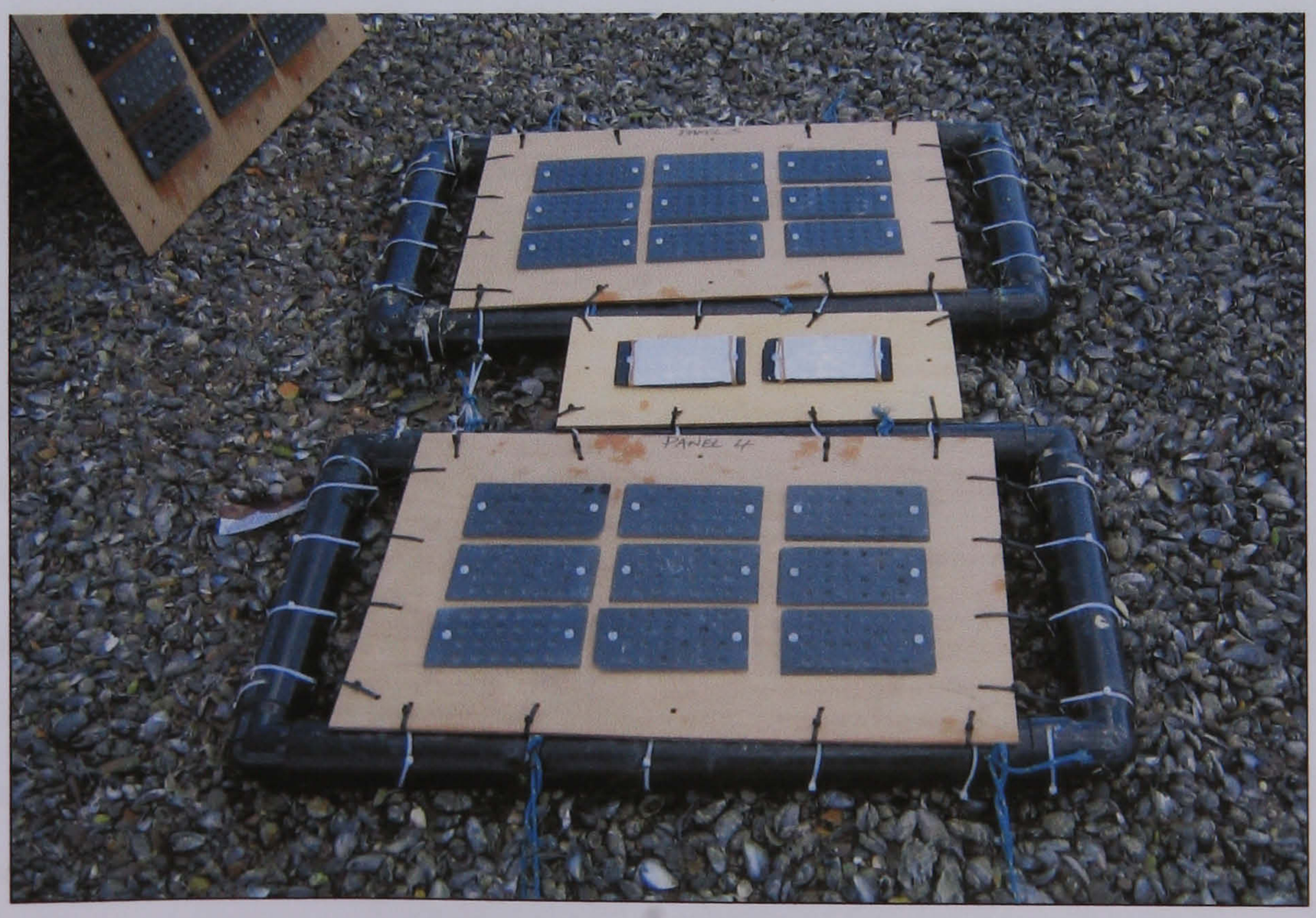



Figure 7.18: Photograph of the experiment panels in situ at the Great Bull Hill

## 7.3 Results

### 7.3.1 *Semibalanus balanoides*

#### Trial experiments 2002

**Slate substrate**

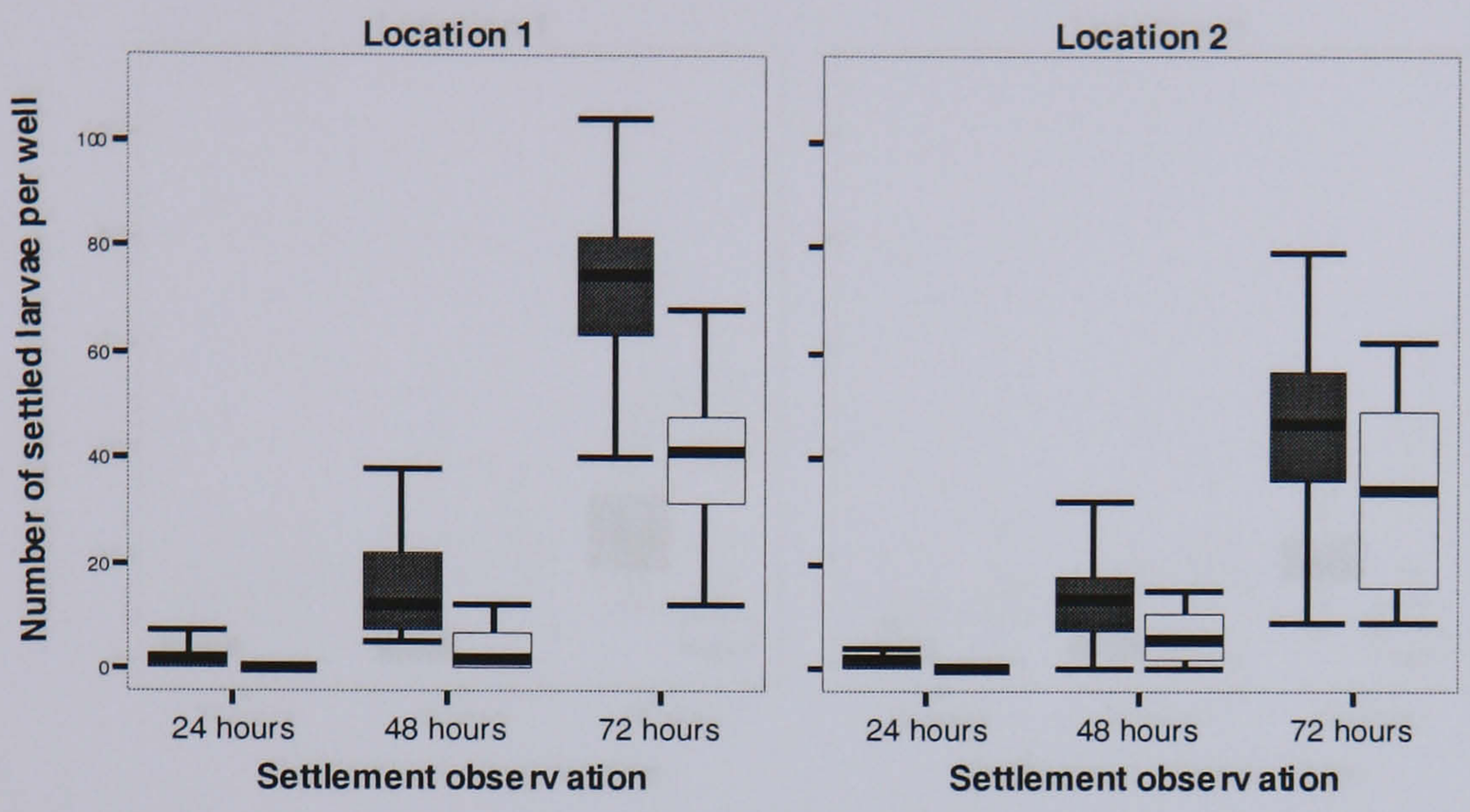
The slates were judged to be both durable and suitable for the experiments. One slate was lost and a second was broken during heavy sea conditions on the final day of Experiment SBF1.2, otherwise the slates remained intact and were re-useable. The majority of settlement occurred in the wells of the slates; a small number of larvae were observed around the edges, and none settled on the flat surface between wells. Figure 7.19 shows *S. balanoides* settlement in a conspecific-treated well of a slate panel.

**Experiment results**

Figure 7.20 illustrates the results of Experiments SBF1.1, and Figure 7.21 those of Experiment SBF1.2. Overall, settlement was higher during Experiment SBF1.1 than SBF1.2. Figure 7.22 illustrates the results of Experiment SBF1.3. Kolmogorov-Smirnov statistics indicated that the raw data of Experiments SBF1.1 and SBF1.2 were not normally distributed, but conversely the distribution of the raw data of Experiment SBF1.3 was normal. All transformations improved the distribution of certain data sets, and overall the square root transformation was most successful. Levene's statistics indicated heteroscedasticity. Table 7.2 summarises the results of the Mann-Whitney U tests that were carried out on the 24-hour data of the experiments. The results of Experiments SBF1.1 and SBF1.2 were similar and reached the same conclusions on significance. The analysis of the slates with both conspecific SF-treated and blank control wells identified that the higher settlement in the conspecific wells was significantly different to that of the control ($P < 0.001$), and the comparison of slates with treated wells to those that were completely blank identified that the total settlement on the slates was significantly different ($P = 0.003$). Thus, SF used in this experimental


Figure 7.19: Photograph illustrating *S. balanoides* settlement in a well treated with conspecific SF. The photograph was taken after 72 hours exposure on the shore. Many larvae have metamorphosed, while newly-settled cyprids are situated mainly towards the left and right in the image.

(a)




(b)

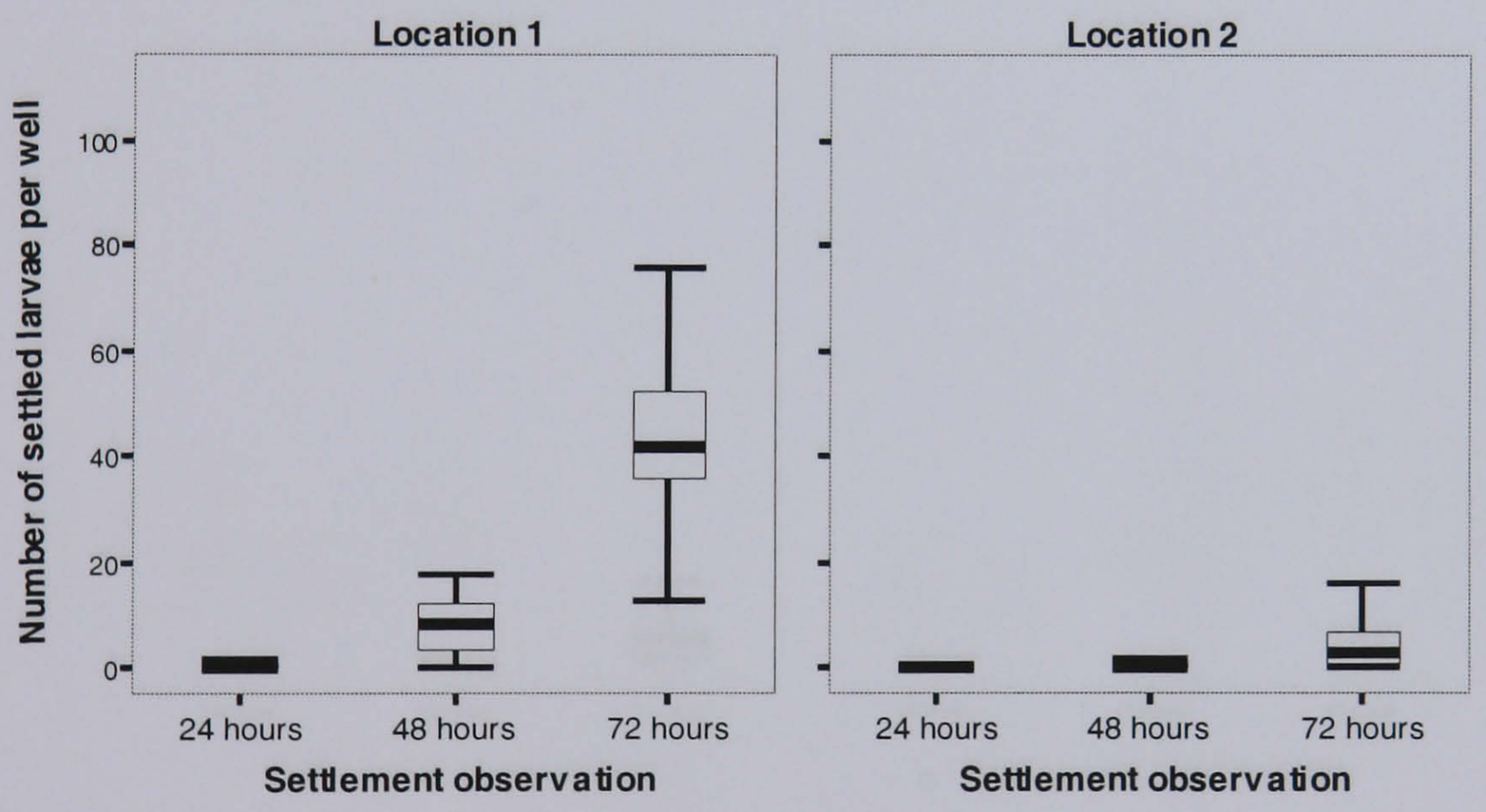
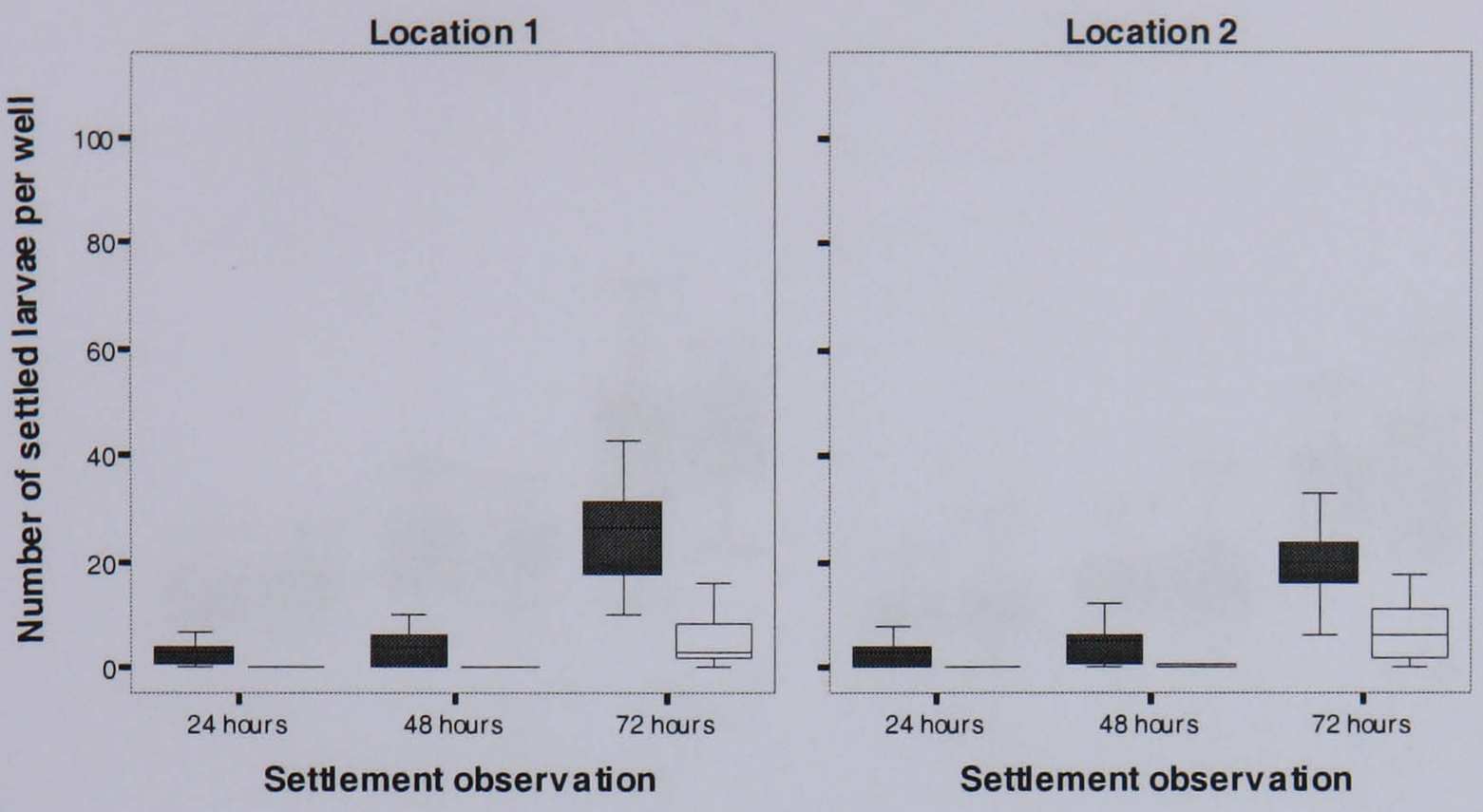



Figure 7.20: The effect of conspecific SF on settlement by *S. balanoides* cyprids. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N= 12 on treated/blank slates and 24 on blank slates. Slates have (a) both *S. balanoides* SF treated and blank control wells and (b) blank control wells only. (Experiment SBF1.1 2002)

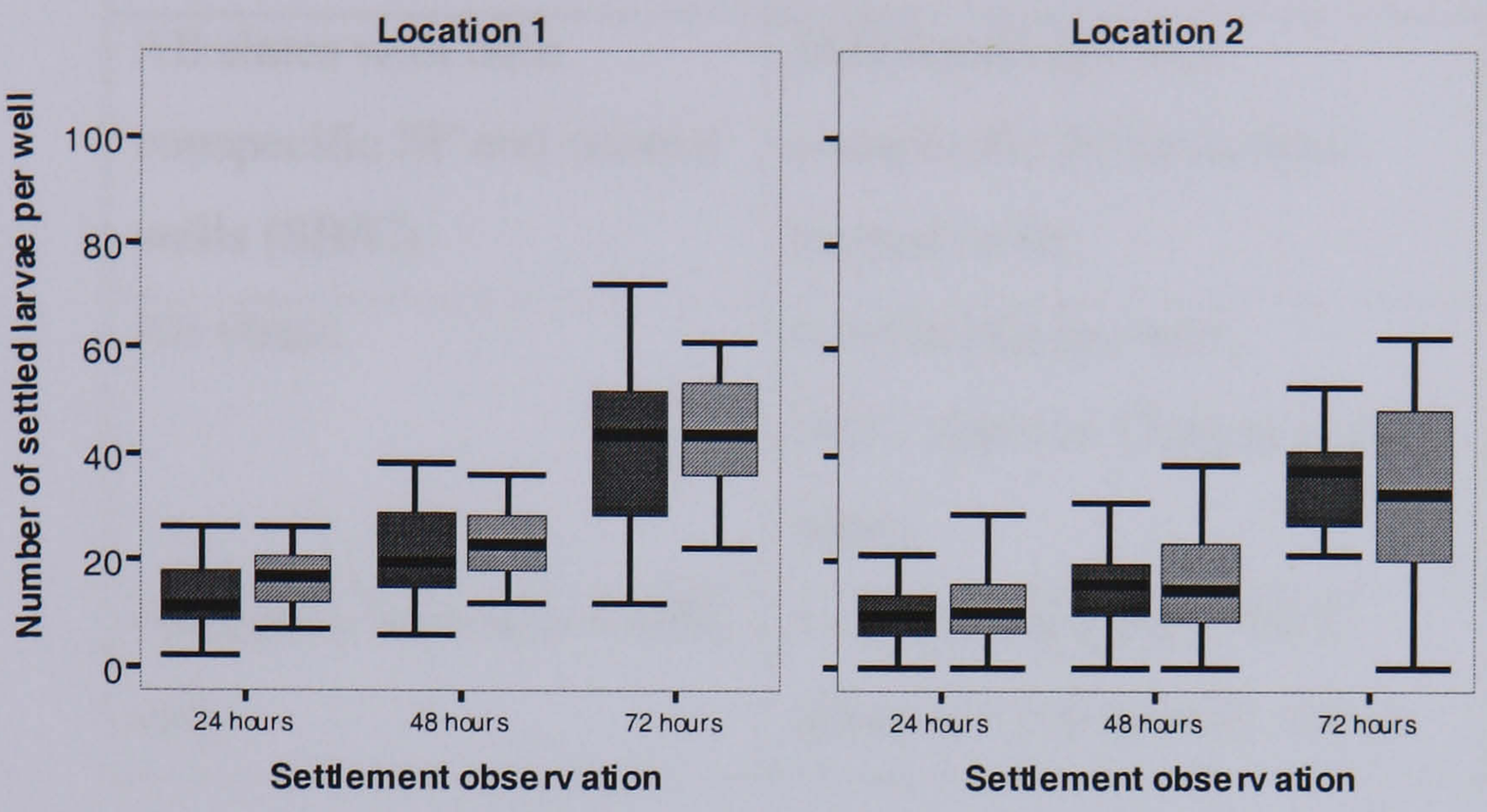
(a)




(b)


Figure 7.21: The effect of conspecific SF on settlement by *S. balanoides* cyprids. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 12 on treated /blank slates and 24 on blank slates. Slates have (a) both *S. balanoides* SF treated and blank control wells and (b) blank control wells only. (Experiment SBF1.2 2002)

(a)




(b)

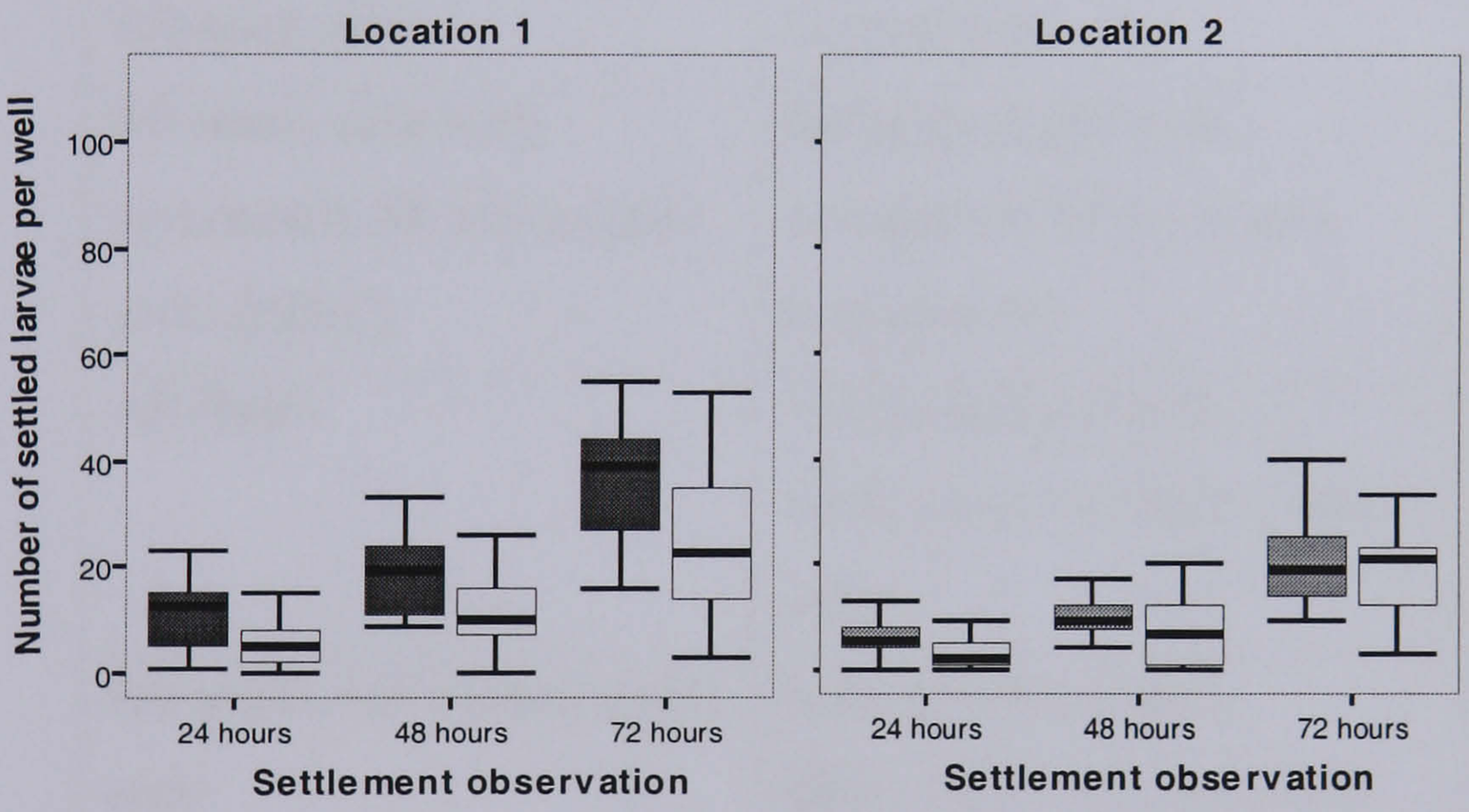



Figure 7.22: The effect of con- and allo-specific SF on settlement by *S. balanoides* cyprids. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 12. Slates have (a) both *S. balanoides* and *E. modestus* SF treated wells and (b) both *S. balanoides* SF treated and blank control wells (Location 1), or both *E. modestus* SF treated and blank control wells (Location 2) (Experiment SBF1.3 2002)

(a)

| Selected slates | Comparison | P value |
|---|---|---|
| All slates with both conspecific SF and control wells (SB/C) | Individuals per well, conspecific SF to control treated wells | $P < 0.001$ |
| All slates | Individuals per well, SB/C slates to Control only slates | $P = 0.003$ |
| All slates, but control wells only | Individuals per well, SB/C slates to Control only slates | $P = 0.138$ |

(b)

| Selected slates | Comparison | P value |
|---|---|---|
| All slates with both conspecific SF and control wells (SB/C) | Individuals per well, conspecific SF to control treated wells | $P < 0.001$ |
| All slates | Individuals per well, SB/C slates to Control only slates | $P < 0.001$ |
| All slates, but control wells only | Individuals per well, SB/C slates to Control only slates | $P = 0.331$ |

Table 7.2: Summary results (P values) of 24-hour settlement comparisons between selected data sets (Experiments SBF1.1-1.3), using Mann-Whitney U tests. $P<0.05$ indicates a significant difference in settlement between data types; (a) Experiment SBF1.1, (b) Experiment SBF1.2 and (c) Experiment SBF1.3.

(c)

| Selected slates | Comparison | P value |
|---|---|---|
| All slates with both conspecific and *E. modestus* SF wells (SB/EM) | Individuals per well, conspecific to allospecific SF treated wells | P = 0.152 |
| All slates with both conspecific SF and control wells (SB/C) | Individuals per well, conspecific SF to control treated wells | P = 0.003 |
| All slates with both *E. modestus* SF and control wells (EM/C) | Individuals per well, allospecific SF to control treated wells | P = 0.003 |
| All slates at Location 1 | Individuals per well, EM/SB to SB/C slates | P = 0.001 |
| All slates at Location 2 | Individuals per well, EM/SB to EM/C slates | P < 0.001 |
| Location 1 slates with both conspecific SF and control wells (SB/C) and Location 2 slates with *E. modestus* SF and control wells (EM/C) | Individuals per well, SB/C to EM/C slates | P = 0.012 |
| Location 1 and location 2 as described above, but control wells only | Individuals per well, SB/C to EM/C slates | P = 0.079 |

Table 7.2 (continued): Mann-Whitney U tests Experiments SBF1.1-1.3 (2002); (c) Experiment SBF1.3.

design did induce settlement. When control wells only were selected, there was no significant difference between those on the partially treated slates and the blank slates. Thus, close proximity of a SF treated well did not adversely influence control settlement. For Experiment SBF1.3, settlement on con- and allo-specific treated wells on dual-SF treated slates was not significantly different, i.e. a larval preference for conspecific SF was not observed. On slates with a single SF treatment, settlement was significantly higher for both the conspecific and the allospecific treated wells compared to the control ($P < 0.001$ for both) i.e. larvae discriminated. Comparisons of overall settlement on slates identified that, at Location 1, settlement on the dual-SF treated slates was significantly different to the slates with conspecific SF and control wells ($P = 0.003$), and similarly at Location 2 settlement on the dual treated slates was significantly different to the slates with allospecific SF and blank wells ($P < 0.001$). The comparison of settlement on slates with conspecific SF and control wells at Location 1 to their counterpart with allospecific SF at Location 2 was significantly different ($P = 0.012$), though when only the blank wells of these slates were selected the settlement was not significantly different. This suggested that the larvae preferred conspecific SF to the allospecific, and is contrary to the direct statistical comparison.

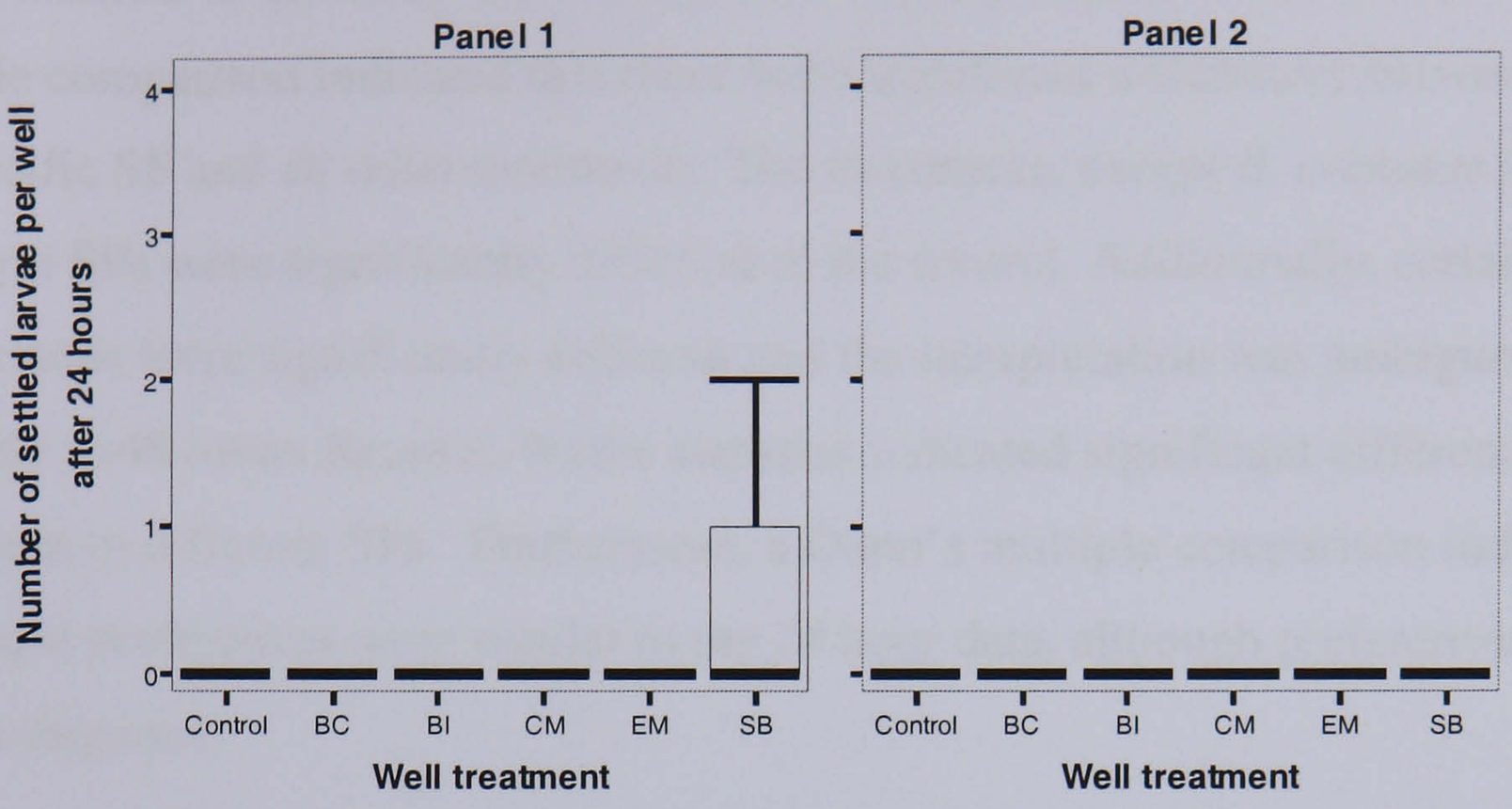
## Experiments 2003

Settlement was low on all panels in all experiments. The results of Experiment SBF2.1 and Experiment SBF2.3 were exceptionally low, with less than 10 cyprids panel$^{-1}$ throughout, and additional analyses of these results were not undertaken.

### Experiment SBF2.2

Settlement was low varying between 0 to 1 cyprid well$^{-1}$ dependent on treatment and experiment day. Generally, settlement was at a similar level on both panels. The exception was the May 13$^{th}$ assay, where Panel 2 settlement was lower than Panel 1. Settlement preferences were similar in each assay. Figure 7.23 illustrates 24-hour settlement. Settlement was highest on *S. balanoides* SF-treated wells, followed by *E. modestus,* then *B. improvisus* and *B. crenatus* SFs. Settlement on *C. montagui* SF was

(a)




(b)

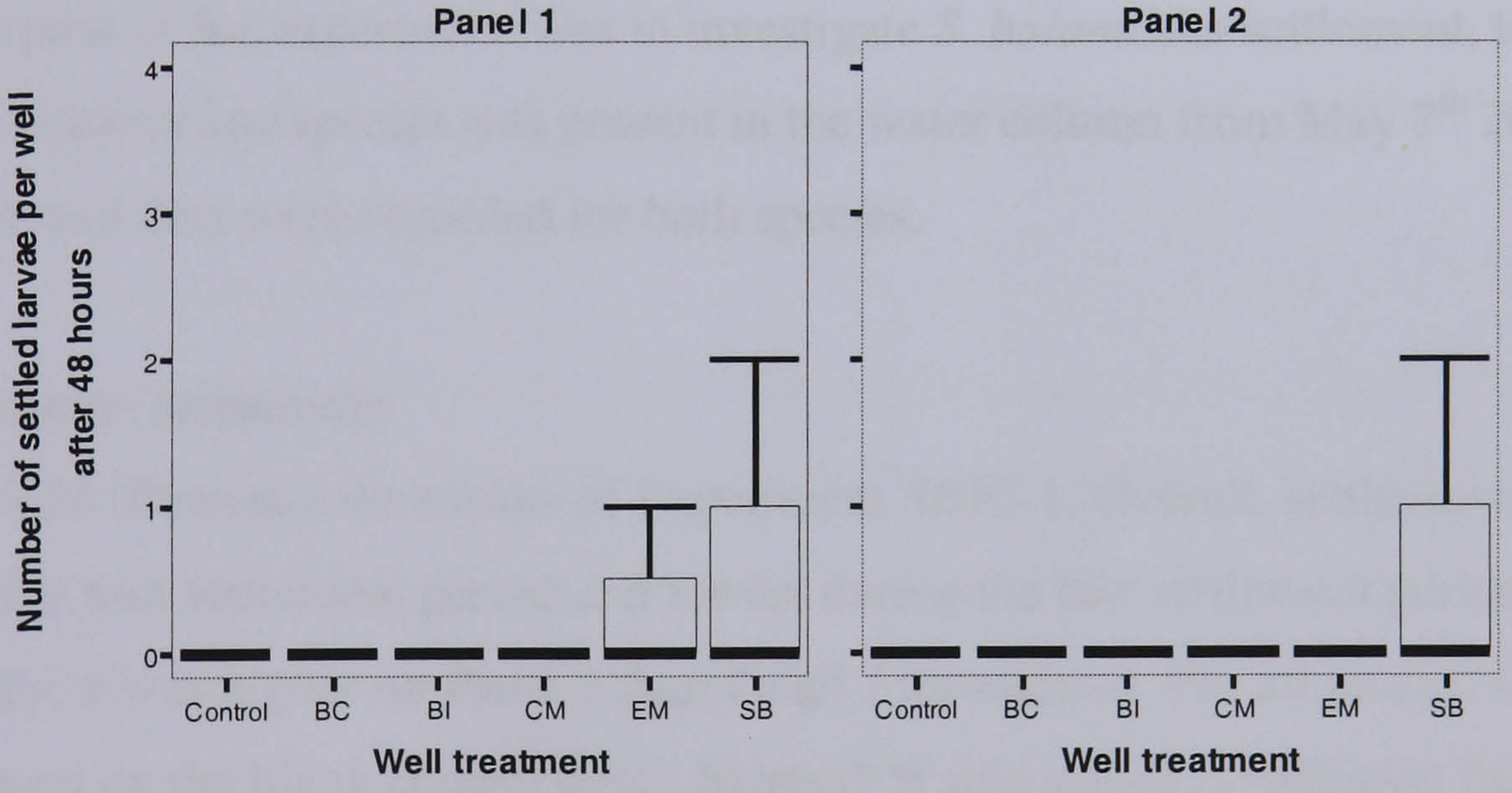



Figure 7.23: The effect of con- and allo-specific SF on settlement well$^{-1}$ by *S. balanoides* cyprids in field conditions; (a) settlement after 24 hours and (b) settlement after 48 hours. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 36 panel$^{-1}$. BC = *B. crenatus*; BI = *B. improvisus*: CM = *C. montagui*: EM = *E. modestus*;
SB = *S. balanoides*. (Experiment SBF2.2 2003)

lower, with control settlement the lowest. Kruskal-Wallis statistics indicated that 24-hour settlement to different SFs was significantly different ($P<0.001$). A Dunn's multiple comparison indicated that there were significant differences between conspecific SF and all other treatments. The treatments, except *B. crenatus* and *C. montagui* SFs were significantly different to the control. Additionally, certain other comparisons were significantly different and the interpretation was ambiguous. Similarly at 48 hours Kruskal-Wallis statistics indicated significant differences in settlement to different SFs. Furthermore, a Dunn's multiple comparison indicated that settlement preferences were similar to the 24 hour data, although preferences continued to be ambiguous.

## Full-scale experiments 2004

### Experiment SBF3.1 (Multiple cue assays)

The purpose of the experiment was to investigate *S. balanoides* settlement, though a second unidentified species was present in the water column from May 7$^{th}$ 2004 onwards and data were recorded for both species.

#### *Semibalanus balanoides*

Figure 7.24 illustrates the results of Experiment SBF3.1. Overall, settlement was highest during the mid settlement period and lowest during the late settlement period, and generally, it was higher on Panel 1 than Panel 2 throughout. For all assays, settlement was lowest on the blank control wells. Kruskal-Wallis statistics indicated that 24 hour settlement to different SFs was significantly different ($P<0.001$) for each period of 5 days (April 28$^{th}$ to May 2$^{nd}$, May 7$^{th}$ to May 11$^{th}$ and May 22$^{nd}$ to May 27$^{th}$). A Dunn's multiple comparison indicated that settlement on all SF treatments was significantly different to the control. Additionally, settlement on conspecific SF was significantly different to all other treatments in the early and late 5 day periods, though in the mid period, settlement on conspecific and *E. modestus* SF were equal. Certain other comparisons were also significantly different, though there were differences between the 5 day periods, as follows:-

1. Early settlement season (April 28th to May 3rd 2004):

*S. balanoides* > *E. modestus* > (*B. crenatus* = *B. improvisus* = *C. montagui*) > Control;

2. Mid settlement season (May 7th to May 12th 2004):

(*S. balanoides* = *E. modestus*) > (*B. improvisus* = *B. crenatus* = *C. montagui*) > Control;

3. Late settlement season (May 22nd to May 27th 2004):

*S. balanoides*> *E. modestus* > (*B. improvisus* = *B. crenatus*) > *C. montagui*) > Control,

where '>" indicates a significant preference, and '=' indicates no significant difference.

The rank of the preferred SF treatment was similar throughout. Settlement was highest on conspecific SF, followed by that of *E. modestus*, then *B. improvisus* or *B. crenatus*, then *C. montagui*, with the control treatment being the least preferred. When all data were combined, conspecific settlement was significantly higher than that of *E. modestus*, which in turn was significantly higher than other SF treatments. Settlement on *B. improvisus, B. crenatus* and *C. montagui* SFs was equal and significantly higher than the control; in summary:-

*S. balanoides* > *E. modestus* > (*B. crenatus* = *B. improvisus* = *C. montagui*) > Control.

**Unidentified species**

Figure 7.25 illustrates the results of the mid and late settlement periods of Experiment SBF3.1 for the unidentified species. Settlement by this species was lower than that of *S. balanoides* overall. Settlement was higher on Panel 2 than Panel 1, and was higher during mid settlement period than the late. Highest settlement occurred on wells treated with *B. improvisus* SF throughout. Kruskal-Wallis statistics indicated that 24 hour settlement to different SFs was significantly different ($P<0.001$) for mid and late settlement periods separately ($P < 0.001$). A Dunn's multiple comparison indicated that settlement on all SF treatments was significantly higher than the control. Additionally, settlement on *B. improvisus* SF was significantly higher than all other SF treatments and no other SF combinations were significantly different, in summary:-

*B. improvisus* > (*B. crenatus* = *C. montagui* = *E. modestus* = *S. balanoides*) > Control.

**Experiment SBF3.2 (Two SF assays)**

Figure 7.26 illustrates the results of Experiment SBF3.2. Panel 1 and Panel 2 results were similar throughout. In all assays, the control settlement was lower than both of the SF treatments. Kruskal-Wallis statistics indicated that 24 hour settlement to different SFs was significantly different for each assay separately ($P < 0.001$). Dunn's multiple comparisons indicated that all SF treatments had significantly higher settlement than the control. While settlement was equal between *S. balanoides* SF and that of *E. modestus*, settlement was significantly higher on the conspecific treatment compared to *B. crenatus*, *B. improvisus* and *C. montagui* SFs.

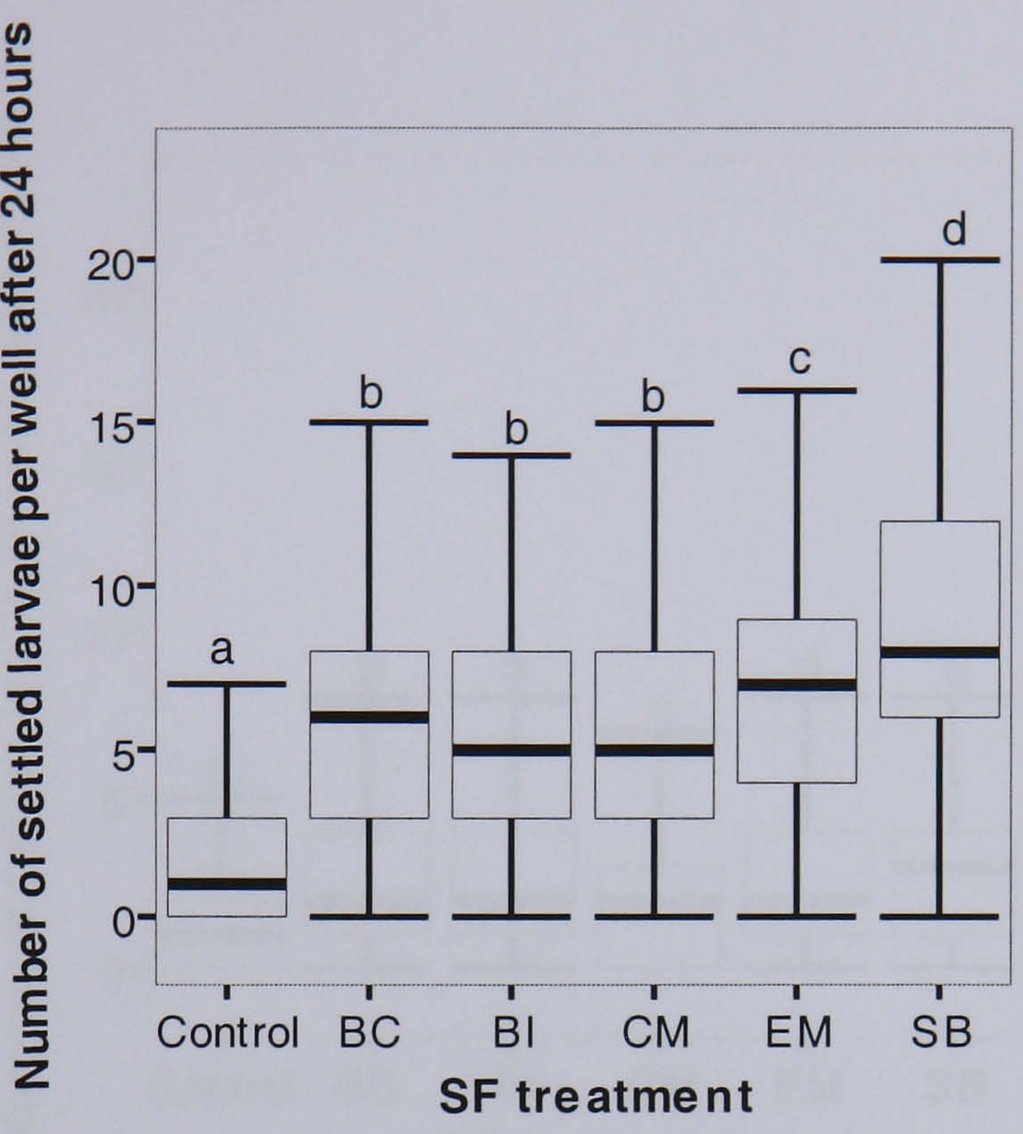
**Experiment SBF3.3 (Aged slates assays)**

Figure 7.27 illustrates the results of Experiment SBF3.3. Settlement was similar for Panel 1 and Panel 2, except for the May 2nd assay. Mann Whitney U statistics indicated that 24 hour settlement to different well treatments, i.e. conspecific and control, was significantly different for each assay separately ($P < 0.001$). Kruskal-Wallis statistics indicated no significant difference in settlement between slate types.

**Experiment SBF3.4 (Nitrocellulose membrane assay trial)**

Figure 7.28 shows the results of Experiment SBF3.4 showing settlement on the nitrocellulose membranes. Overall, settlement was highest on May 8th, followed by May 10th, and lowest on May 22nd 2004. In each assay, settlement was higher on SF-treated spots than on the control and, in Assay 3, there was no settlement on the BSA control. Mann-Whitney U statistics indicated that settlement on conspecific SF treated spots was significantly higher than on the controls for all assays ($P < 0.001$, May 8th; 0.000, May 10th; 0.001, May 22nd).

(a)




(b)

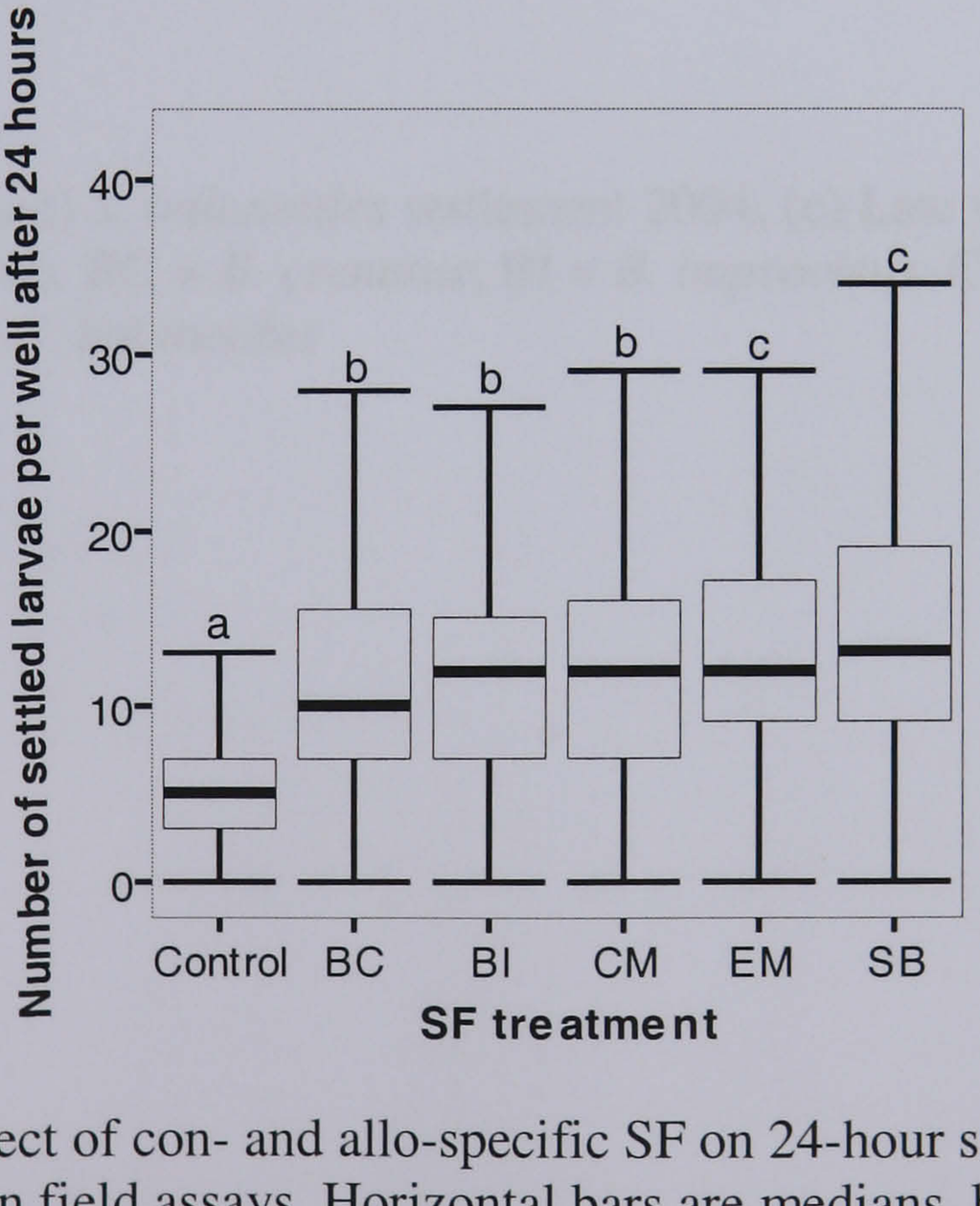



Figure 7.24: The effect of con- and allo-specific SF on 24-hour settlement by *S. balanoides* cyprids in field assays. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 36 panel $^{-1}$. Different letters above bars indicate significant differences, $P \leq 0.05$; (a) Early settlement season, (April 28$^{th}$ to May 3$^{rd}$ 2004), (b) Mid settlement season (May 7$^{th}$ to May 12$^{th}$ 2004) and (c) Late settlement season (May 22$^{nd}$ to May 27$^{th}$ 2004). BC = *B. crenatus*; BI = *B. improvisus*: CM = *C. montagui*: EM = *E. modestus*; SB = *S. balanoides.* (Experiment SBF3.1 2004)

(c)

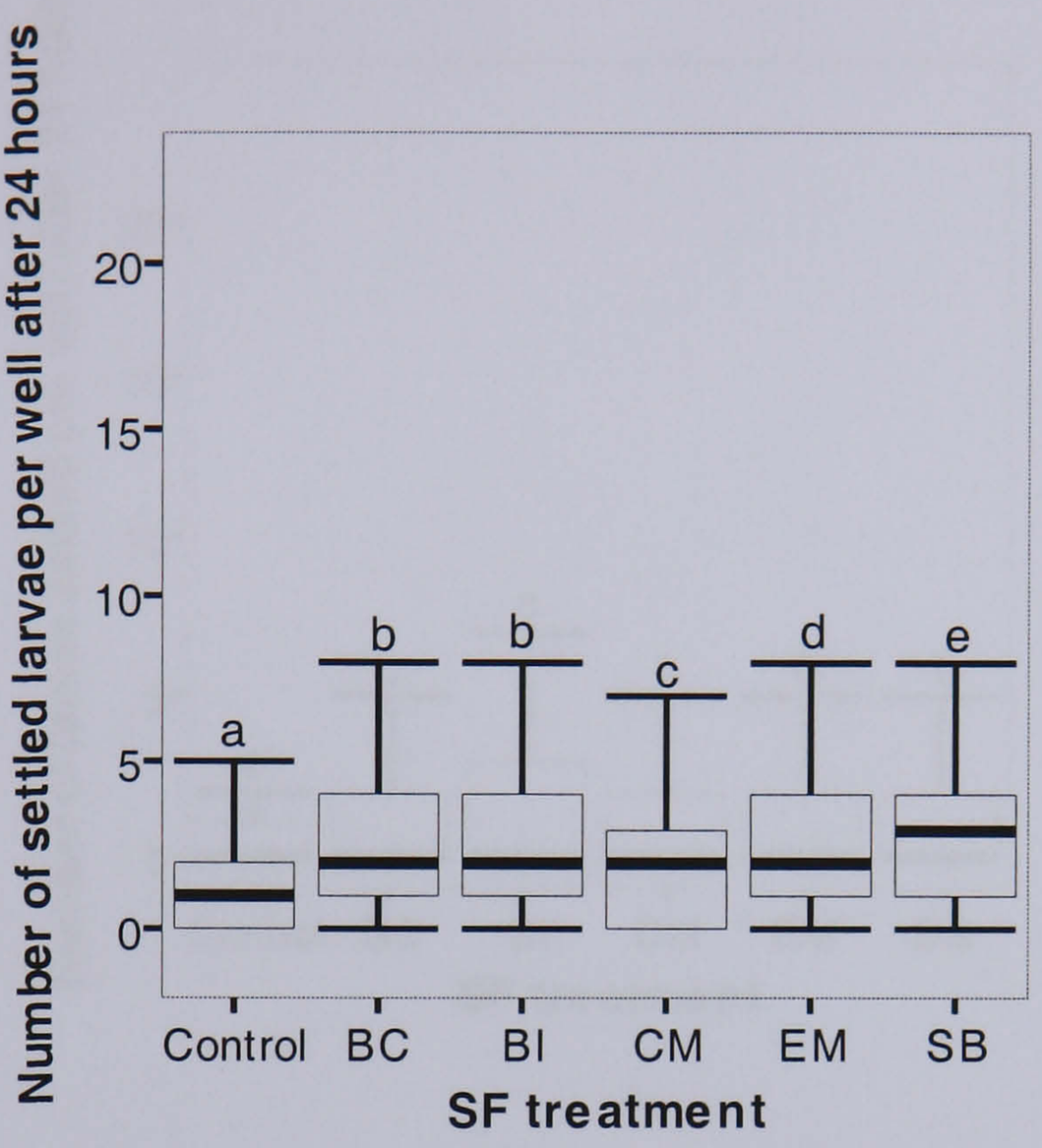
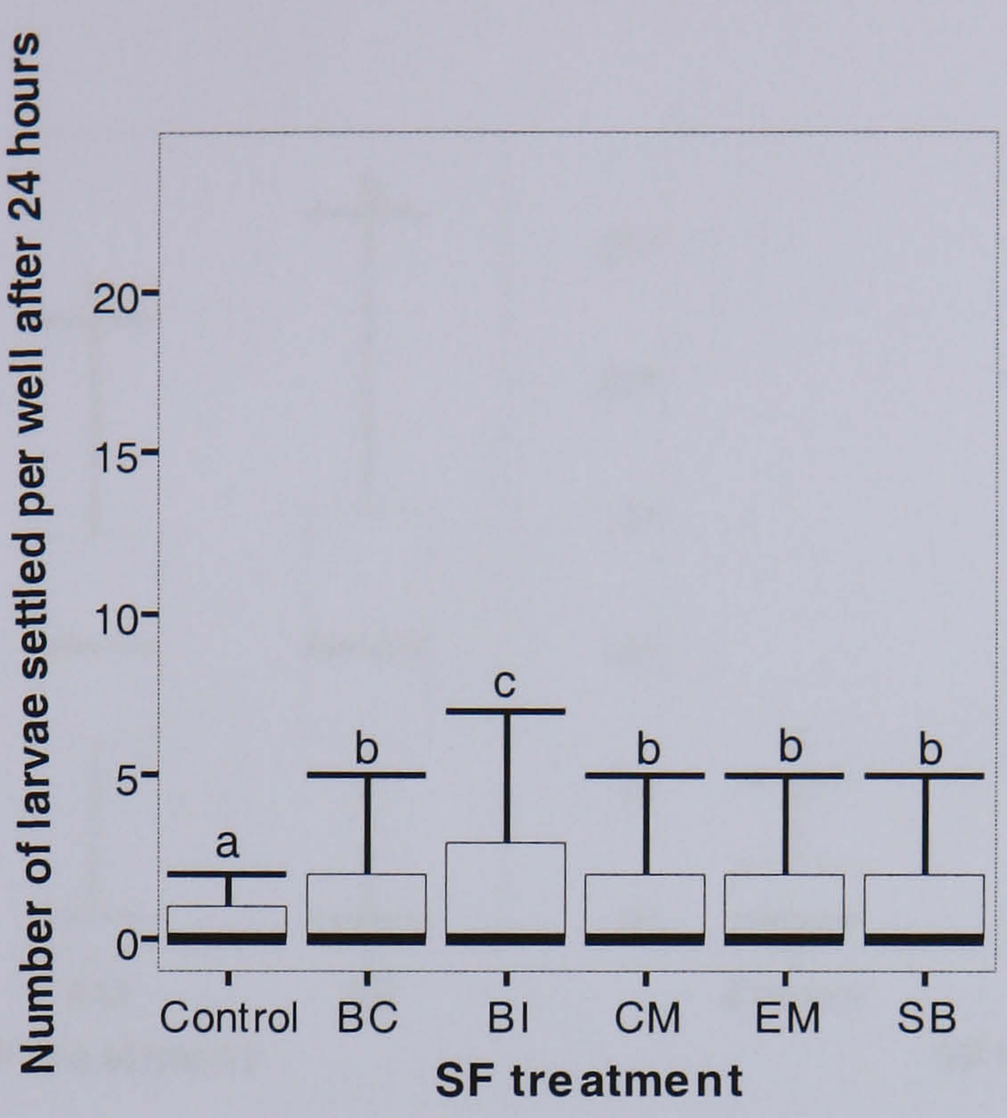



Figure 7.24 (continued) *S. balanoides* settlement 2004; (c) Late settlement season (May 22nd to May 27th 2004). BC = *B. crenatus*; BI = *B. improvisus*: CM = *C. montagui*: EM = *E. modestus*; SB = *S. balanoides*

(a)




(b)

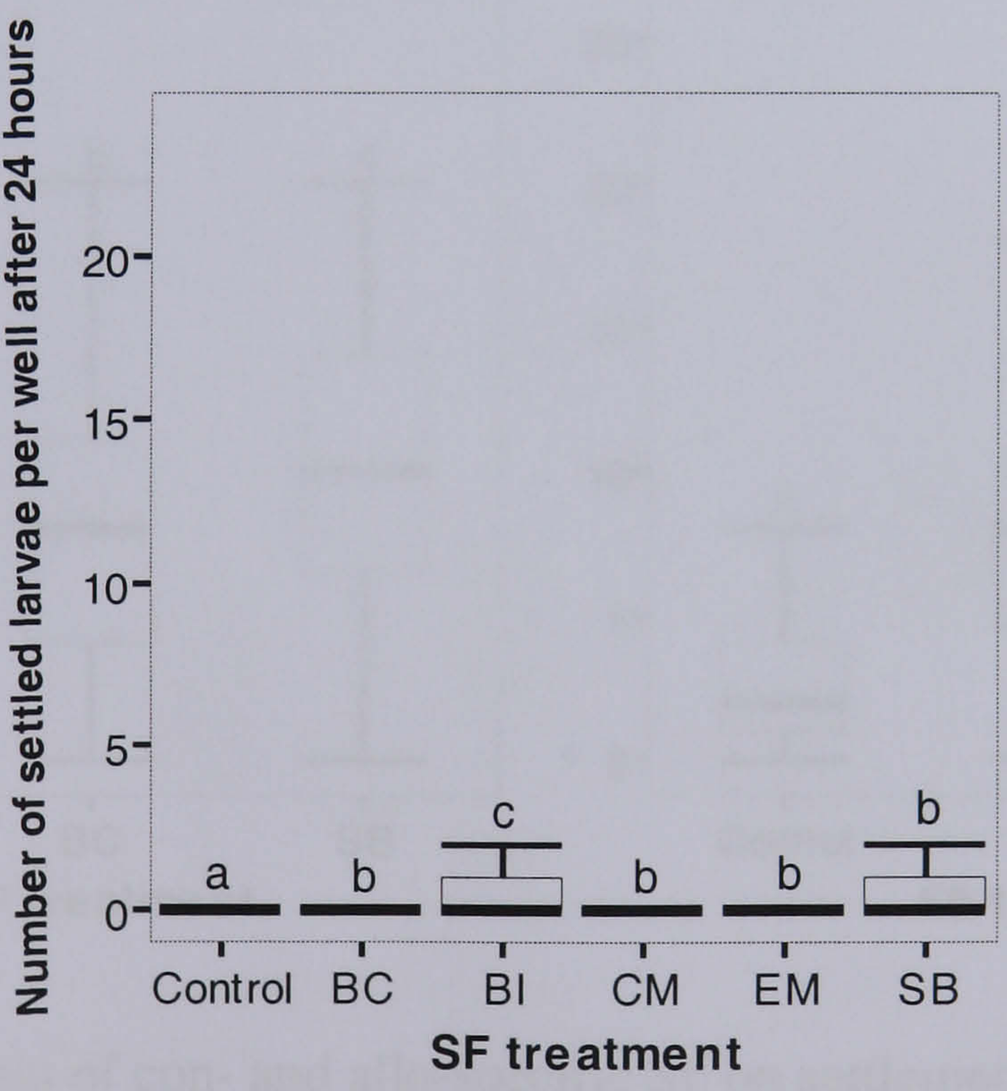



Figure 7.25: The effect of con- and allo-specific SF on 24-hour settlement by the unidentified species in field assays. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 36 panel $^{-1}$. Different letters above bars indicate significant differences, $P \leq 0.05$; (a) Mid settlement season (May 7$^{th}$ to May 12$^{th}$ 2004) and (b) Late settlement season (May 22$^{nd}$ to May 27$^{th}$ 2004). BC = *B. crenatus*; BI = *B. improvisus*: CM = *C. montagui*: EM = *E. modestus*; SB = *S. balanoides*. (Experiment SBF3.1 2004)

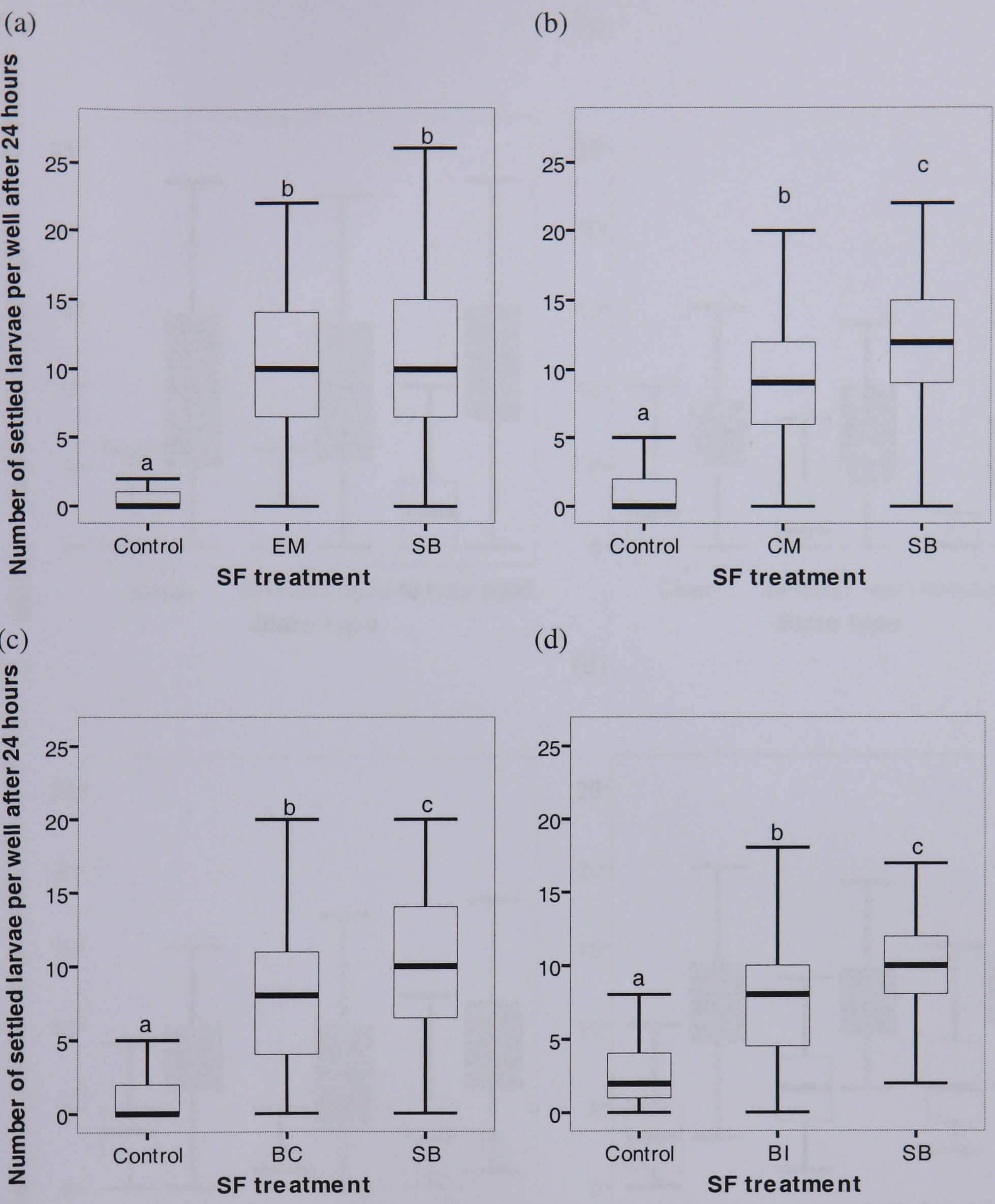



Figure 7.26: The effect of con- and allo-specific SF on settlement by *S. balanoides* cyprids Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 72 panel$^{-1}$; (a) May 3$^{rd}$ 2004 – Location 1: *S. balanoides* (SB)and *E. modestus* (EM) SFs and a blank control, (b) May 3$^{rd}$ 2004 – Location 2: *S. balanoides* (SB) and *C. montagui* (CM) SFs, and a blank control, (c) May 4$^{th}$ 2004 – Location 1: *S. balanoides* (SB) and *B. crenatus* (BC) SFs and a blank control and (d) ) May 4$^{th}$ 2004 – Location 2: *S. balanoides* (SB) and *B. improvisus* (BI) SFs and a blank control (Experiment SBF3.2 2004)

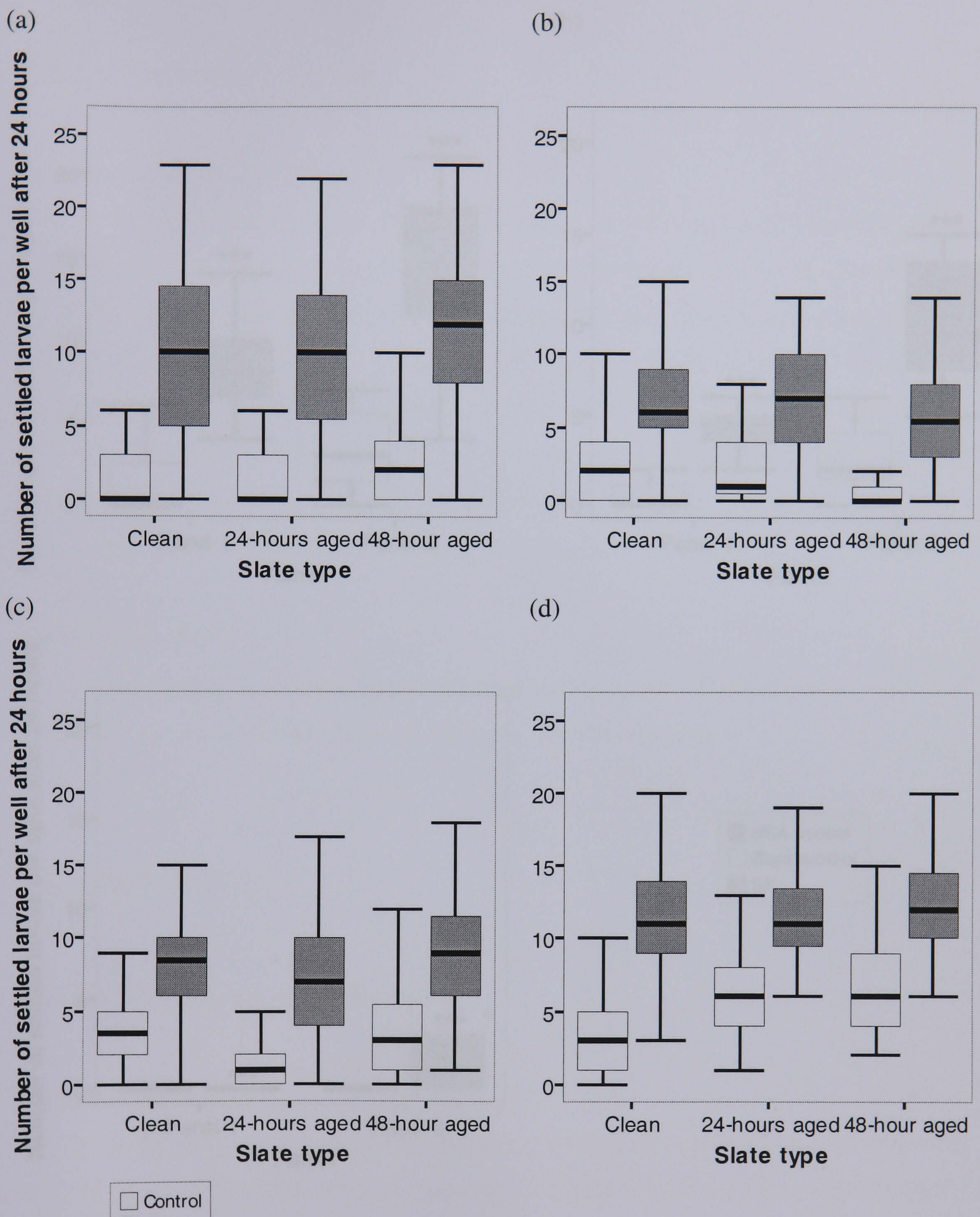



Figure 7.27: The effect of conspecific SF on settlement by *S. balanoides* cyprids on slates that were aged in seawater. The 3 slate types were: 1.Not aged, 2. 24-hours aged and 3. 48-hours aged. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 108 panel$^{-1}$; (a) April 29$^{th}$ 2004 (Assay 1), (b) May 2$^{nd}$ 2004 (Assay 2), (c) May 7$^{th}$ 2004 (Assay 3), (d) May 11$^{th}$ 2004 (Assay 4). (Experiment SBF3.3 2004)

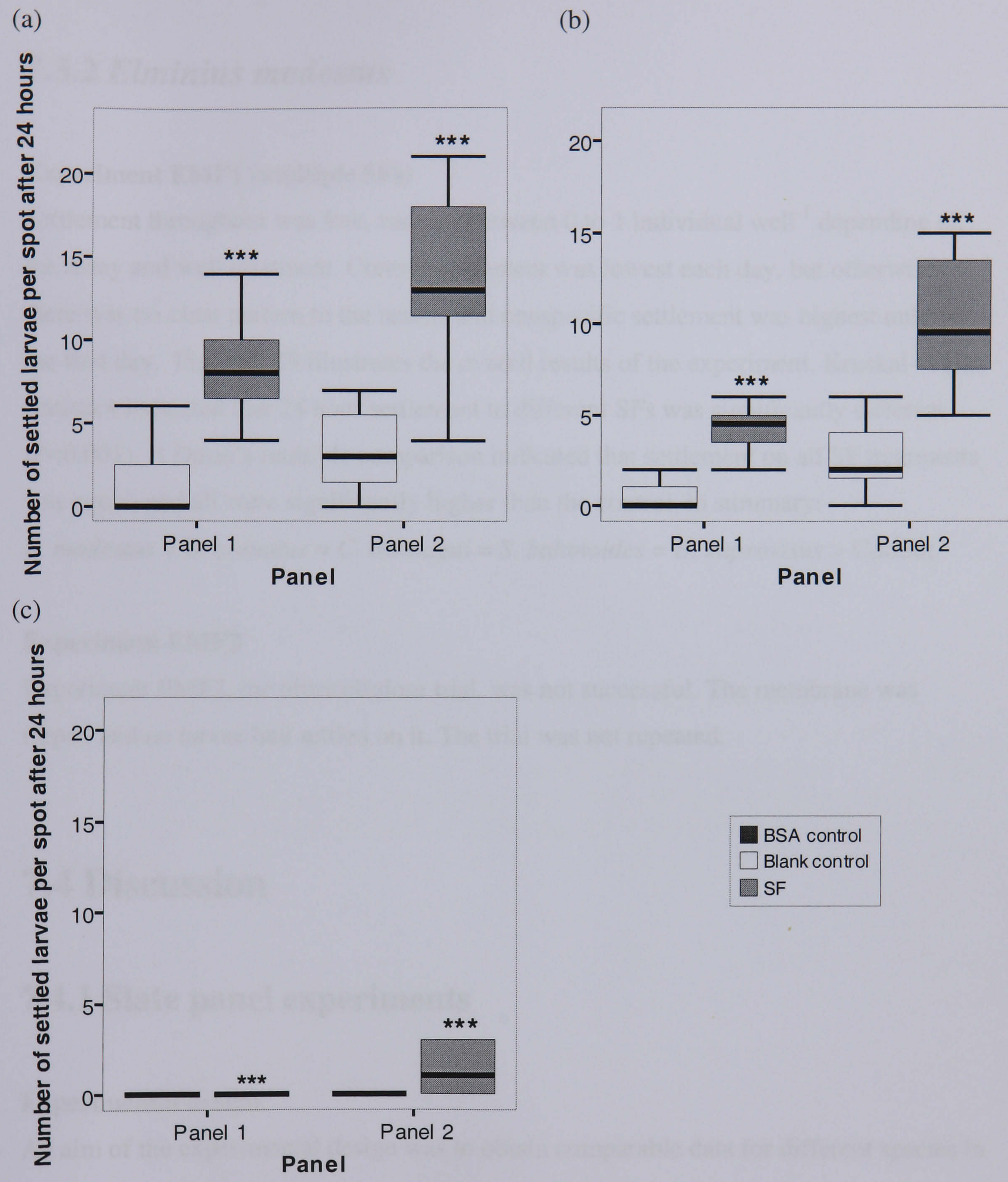



Figure 7.28: The effect of conspecific SF on settlement by *S. balanoides* cyprids in the nitrocellulose membrane field assay. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 12; (a) May 8th 2004, (b) May 10th 2004, (c) May 22nd 2004. Control treatment is buffer for May 8th and May 10th assay, and BSA for May 22nd assay. ***P <0.005 (Experiment SBF3.4 2004)

### 7.3.2 *Elminius modestus*

**Experiment EMF1 (multiple SFs)**

Settlement throughout was low, varying between 0 to 1 individual well$^{-1}$ depending on the assay and well treatment. Control settlement was lowest each day, but otherwise there was no clear pattern to the results and conspecific settlement was highest only on the first day. Figure 7.29 illustrates the overall results of the experiment. Kruskal-Wallis statistics indicated that 24 hour settlement to different SFs was significantly different ($P<0.001$). A Dunn's multiple comparison indicated that settlement on all SF treatments was equal, and all were significantly higher than the control, in summary:-
*E. modestus* = *B. crenatus* = *C. montagui* = *S. balanoides* = *B. improvisus* > Control.

**Experiment EMF2**

Experiment EMF2, the nitrocellulose trial, was not successful. The membrane was ripped and no larvae had settled on it. The trial was not repeated.

## 7.4 Discussion

### 7.4.1 Slate panel experiments

**Experimental design**

An aim of the experimental design was to obtain comparable data for different species in the field, and also to enable a comparison between field and laboratory results. Barnett and Crisp (1979) argued that laboratory assays were the only way to effectively compare settlement behaviour of different barnacle species. The basis of the field data was post settlement counts directly on rocks and they considered that comparisons of two species may have been biased by differences in survival that resulted from different settlement

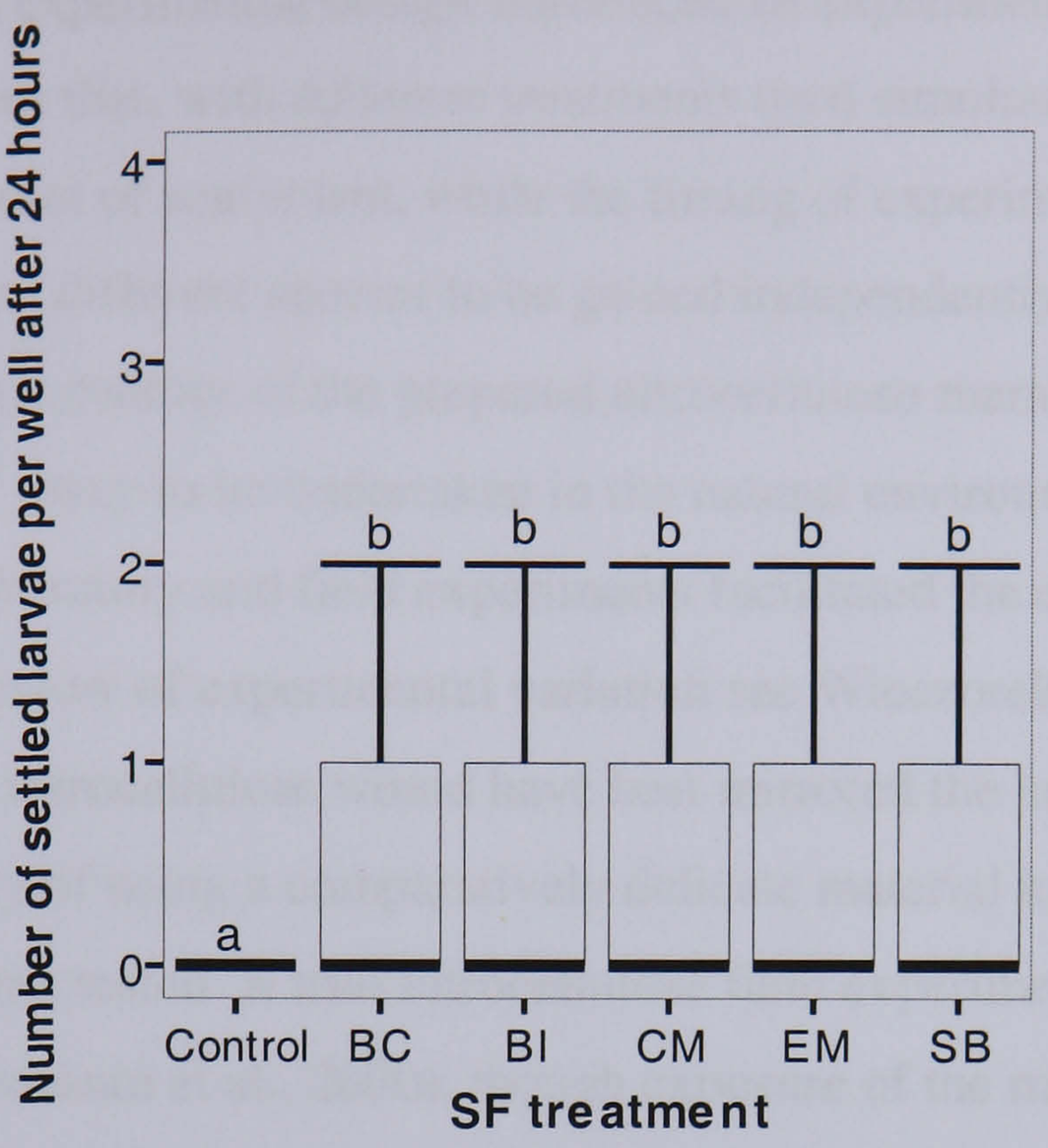



Figure 7.29: The effect of con- and allo-specific SF on 24-hour settlement by *E. modestus* cyprids in field assays. Horizontal bars are medians, boxes indicate 1X interquartile ranges and vertical bars 1.5X interquartile ranges. N = 36 panel $^{-1}$. Different letters above bars indicate significant differences, P≤0.05. BC = *B. crenatus*; BI = *B. improvisus*; CM = *C. montagui*; EM = *E. modestus*; SB = *S. balanoides*. (Experiment EMF1 2004)

dates. The present experimental design introduced an experimental substrate into the natural environment that, with different treatments used simultaneously, captured cyprid behaviour at the point of settlement, while the timing of experiments enabled comparable data for different species to be gained independently. The slate design mirrored the surface contour of the prepared nitrocellulose membrane (Figure 2.3) and enabled a 'choice' assay to be undertaken in the natural environment. The uniformity of design between laboratory and field experiments facilitated the effective comparison of such results (for review of experimental variation see Wieczorek and Todd, 1998). Ideally, the use of nitrocellulose would have best mirrored the laboratory method, though the viability of using a comparatively delicate material in the natural environment was not tested. A trial nitrocellulose field experiment had been undertaken successfully (Matsumura et al., 2000), though exposure of the membrane was limited to 30 minutes and its durability over a sustained period had yet to be established. Additionally, the cost for full scale experiments was considered prohibitive.

**Slate considerations**

Slate was chosen as it was inexpensive and relatively easy to cut, combined with the fact it was an established effective substrate (e.g. Crisp and Meadows, 1962, 1963). The disadvantage of slate was that, as a natural material, although all panels were cut in the same manner from a single supply (to reduce variability), some differences in surfaces properties of the well surfaces were inherent. However, as many parameters of the shore environment are highly variable at small spatial scales, which may also influence results, the presence of subtle substrate differences was considered an additional factor, rather than a sole perpetrator of bias. Thus, the impact on results specifically due to slate variation was not considered deleterious. However, the replication and distribution of treatments within each experiment was an essential consideration, so that naturally occurring environmental variation did not bias results. Slate panels have been used in the natural environment, though they were smaller than the current design, and were slotted into a supporting panel (A.Clare pers.comm.). Field experiments using large slate panels, similar in size to this design, were unsuccessful due to excessive breakages (J.Delany pers.comm.). Little damage was experienced in the trial experiments, and the

layers of foam and rubber between the slate and the rock were an effective shock absorber.

A concentration of 1 mg $ml^{-1}$ SF was selected to treat the slate wells in accordance with the earlier findings investigating threshold concentrations of SF (Crisp and Meadows, 1962). While this concentration was above the settlement threshold for both *S. balanoides* and *E. modestus*, it induced settlement most effectively. The molecular configuration of the active protein when adsorbed to slate may be very similar at the surface layer to its configuration when bound to the adult epicuticle (Crisp and Meadows, 1962). While the results of previous experiments (e.g. Crisp and Meadows, 1962) have confirmed that the active protein was adsorbed to the slate, other proteins in the mix may not have been similarly attached and, if so, the use of slate may have further purified the protein. Calculations of the amount of SF used, i.e. (prepared volume – waste volume)/ number of wells, indicated that each well was treated with 10.0±0.3 $\mu$g of protein. As a monolayer of protein equates to 1 mg $m^2$ (Crisp and Meadows, 1962), the application of 10 $\mu$g over a well surface area of ca. 0.8 $cm^2$ equates to 125 molecular layers of protein. Furthermore, the activity to SIPC has been shown to be 10-fold that of SF (Matsumura et al., 1998a), and thus if the active protein were 10% of the SF mix, this equates to 12.5 molecular layers. The amount of active protein required to stimulate settlement was very small. There were likely to be small variations in the amount of SF used in each well, due to its manual application using artist's paintbrushes. The variation, like that of the substrate, was considered a further minor source of flux in an environment where parameters were not controlled, with variation over small spatial scales. Again the need for adequate replication was emphasised.

The cleaning of the slates before reuse in further assays was a critical factor to the success of the experiments. The extract, particularly when bound to slate, remained active after extended seawater immersion (Crisp and Meadows, 1962). As a novel random design of well treatments was used for each assay, remnants of previously-applied extracts could confuse the signal of individual wells. Adequate cleaning of the slates between experiments was essential. Crisp and Meadows (1962) determined that

soaking slates in 12% sodium hypochlorite for 15 minutes, or heating them to 270°C for 30 minutes rendered the substance inactive. Thus, the combined action of soaking in 5% sodium hypochlorite for 12 hours, followed by heating to 150°C for 120 minutes was considered sufficient to destroy previously applied extracts. The fact that control settlement was significantly lower in all assays confirmed that cleaning was effective.

**Trial experiments**

The 2002 trial site at Seaham, Co. Durham had the advantages of good larval supply in an apparent *S. balanoides* monoculture, thus reducing the presence of waterborne cues from other barnacle species that could influence settlement behaviour. However, while the trial slates were moderately inconspicuous, the site was unsuitable for full-scale experiments, as it was a small shore with very high human 'traffic', such that tampering with experimental panels, whether curiosity-driven or malicious, was a major concern.

The results of the trial indicate that the method was robust and suitable for the testing of multiple SFs in a single experiment. As anticipated for the trial, the replication was too low for the results to be conclusive. Selective behaviour by larvae may have been masked by low replication in a highly variable environment. Increased replication was required for full scale experiments and parameters were decided in the light of the trial results, as follows: 1) Control wells on all slates were essential. Not only would this indicate if larvae were discriminating, it could be an early indication of poor cleaning of slates in between experiments; 2) All SF treatments should be tested on all slates to remove concern of spatial variation and a latin square design was favoured. The redesign of slates from 24 wells to 36 was considered, so that a 6 x 6 design could be located on a single slate, but the idea was rejected, as larger slates were considered vulnerable to damage. Thus, although latin squares were used, the layout overlapped slates, and arguably the design was a randomised block design; 3) Replication should be increased to take account of both the ambiguous con- and allo-specific comparisons (Experiment SBF1.3) and the fact that 6 treatments (5 SFs plus the control) were to be tested simultaneously. The number of slates was increased by 50% to allow for the earlier ambiguity, and then 3-fold for the increased number of treatments.

**Experiments 2003**

St Andrews Bay was selected, as the shore was extensive and had access from one side only, offering some seclusion to areas furthest away from the point of access. The shore was used successfully with no evidence of visitor intervention. While other barnacle species were evident, such as *B. crenatus* and *E. modestus*, *S. balanoides* was by far the dominant species, such that other barnacle waterborne cues, which could influence settlement behaviour, were likely to be minimal. The number of slates required in full scale experiments prohibited direct attachment to the rocks, a problem that was resolved by the attachment of slates to a panel, and then to a concrete frame (C.Todd pers.comm.). A foam layer between the slates and the panel was used as a shock absorber against wave action, and losses were nil. The concrete frames were intended for horizontal use, and were sufficiently heavy to remain in position during the tidal cycle. They were cumbersome when placed vertically and it was perhaps purely by luck that panels complete with frames were not torn from their rope attachments and lost. The concrete frames were not used for the 2004 experiments.

The 2003 results were ambiguous due to low settlement on the panels.. The cause was a low larval supply at the end of the settlement season. Additionally, the available cyprids were more likely to be 'older', and therefore potentially less discriminating (Crisp and Meadows, 1962), though differences in settlement to the selected cues still occurred. Control settlement was consistently low indicating that cyprids were discriminating. SF-induced settlement was consistently lowest on *C. montagui* treated wells and at times was lower than control treatments, as anticipated by species relatedness (Pérez-Losada et al., 2004). However, the interpretation of the statistical analysis was ambiguous and the results were inconclusive due to low settlement overall.

The absence of settlement on the nitrocellulose membrane may have also been due to low larval supply. Additionally, light-coloured panels have reduced settlement density compared to dark counterparts (J.Phelan pers.comm.) and the white reflective quality of the nitrocellulose was likely to have been a contributory factor. Although settlement was nil, the durability of the material in the natural environment was partially proven. The

choice of location had been relatively sheltered, and the opportunity remained to test the material in a more exposed position.

**Full-scale experiments 2004**

The major advantage of Tentsmuir was its seclusion, which reduced the possibility of passers-by tampering with experimental panels. Additionally, as *S. balanoides* was by far the dominant barnacle species, the effects of other barnacle cues, which could influence behaviour, were likely to be minimal. However, the geographic location of the shore had some drawbacks. The coastline to the south of the site was sandy for several miles, and with local circulating currents moving south to north, larvae reaching Tentsmuir had had no opportunity to settle for several days. Thus, the probability that they were 'older' individuals was increased, and if discriminating ability to different SFs was reduced with increasing age (Crisp and Meadows, 1963), differences in settlement preferences may have been masked. The presence of the second barnacle species provided the opportunity to consider both the settlement behaviour of a further species, as well as the possible interactive effects of two species.

***Semibalanus balanoides***

For each experiment period (early, mid and late settlement season), the ranked position of the different SFs in *S. balanoides* settlement preferences was consistent, other than the interchanging position of *B. crenatus* and *B. improvisus*. The close relatedness of these two species (Pérez-Losada et al., 2004) may explain this. The following statement summarises the overall relatedness (combining morphological and genetic analyses) of *S. balanoides* to the other species:-

(*S .balanoides*) *B. improvisus, B. crenatus*.) *E.modestus*) *C. montagui*)

Perez-Losada et al. (2004).

Allospecific settlement by *S. balanoides* in response to *E. modestus* has long been recognised (e.g. Barnett and Crisp, 1979; Barnett et al., 1979). This experiment allowed the direct comparison of *E. modestus* induced settlement to that of more closely related species. If settlement activity accords with systematic affinity, as first proposed by Knight-Jones (1955), then settlement induced by *B. crenatus* and *B. improvisus* would

be expected to be most similar to the conspecific, followed by that of *E. modestus*, while that induced by *C. montagui* would be less (as indicated by the relatedness of species in the statement above). The ranking and significance of the SFs, overall, suggested that the conservation of an element of *E. modestus* SIPC may have been of greater influence than the systematic affinity of species. The relevance of systematic affinity cannot be disregarded, as larvae did respond to all cues. Furthermore, during the mid settlement period, *B. improvisus*- and *B. crenatus*-induced settlement was significantly higher than that of *C. montagui*, suggesting systematic affinity was influential.

Experiment SBF3.2, in which wells were treated with conspecific and one allospecific SF, was carried out to gain an additional insight to settlement preferences. By limiting SF treatments, the potential influence of cross-inducement, as a result of a larva visiting more than one well, was restricted, with the possibility that differences in preferences may be more easily ascertained. The experiment was carried out over two days immediately after the early settlement season assays of Experiment SBF3.1. Larval supply was increasing, though had not reached a peak (C.Todd pers.comm.). A disadvantage of the experiment design was that assays had different spatial and temporal parameters, such that the results were not directly comparable. However, the level of control and conspecific settlement were separately similar in all assays, suggesting that comparisons may have been valid. The results indicated a settlement association by *S. balanoides* to *E. modestus* that exerts more influence than similarities as a result of the degree of relatedness in *B. crenatus* and *B. improvisus*. The SIPC of *S. balanoides* and *E. modestus* SIPC may have an area of similar recognition.

Experiment SBF3.3, investigating settlement behaviour in the presence and absence of biofilm and SF, simultaneously and separately, was undertaken to consider whether the development of a natural biofilm during the assay had an effect, either facilitatory or inhibitory, on barnacle settlement, and thus only young immature biofilms were tested. The presence of any species' SF, a protein extract, was likely to enhance film development, which may have subsequently masked differences in settlement behaviour between SFs, though control settlement would remain unaffected. Wahl (1989)

described the typical development of substratum epibiosis. Within seconds of immersion in seawater, macromolecules, such as glycoproteins, proteoglucans and polysaccharides, start to adsorb to the surface, providing the opportunity for bacterial colonisation during the first 24 hours, followed by diatoms from the start of day 2 onwards. Thus, while a bacterial film was present on the 24-hour aged slates, some diatoms were also likely to be present on the 48-hour aged slates. As control settlement, with and without a biofilm, and SF settlement, with and without a biofilm, were both not significantly different, the influence of a developing biofilm was considered minimal. Thus, the development of a biofilm during the main experiments would not bias results.

The subtle changes in SF preferences during the mid season period of Experiment SBF3.1 may be linked to increased larval supply. Settlement was increased during this period. Additionally, separate research carried out at the site, investigating larval density, recorded increased larval counts in adjacent traps during this period (C.Todd pers.comm.). Miron et al. (1999) found that *S. balanoides* settlement peaked when numbers of planktonic cyprids were highest, though Olivier et al. (2000) found that *B. amphitrite* settlement counts were poorly correlated to larval supply. With increased intraspecific competition for limited space, the less 'desirable' allospecific locations may have been a good alternative in the presence of many conspecifics. Alternatively, changes to behaviour may have been influenced by the presence of a greater proportion of 'older' individuals. Intraspecific competition for space as a result of increased larval density may have reduced the proportion of larvae settling at early opportunities along the coast, such that when they reached Tentsmuir after several days of inhospitable sandy beaches, many were 'desperate' to settle. Rittschof et al. (1984) showed that older *B. amphitrite* cyprids were less discriminating to SF than young individuals and Crisp and Meadows (1963) reported a decline in specificity with increasing cyprid age. Control settlement was significantly lower throughout the experiment. If ageing influenced SF preferences, an initial partial deterioration of discriminatory ability may have affected choices between species, while the general presence or absence of barnacles remained detectable. The results of the late settlement period indicated that larvae had regained discriminatory ability, which could be due to either reduced

intraspecific competition, or the reappearance of 'younger' more 'choosy' individuals. While larval supply may have been anticipated to have a greater proportion of 'older' cyprids simply because it was later in the season, alternatively 'young' cyprids of late-released, or slow-to-develop, nauplii may have dominated the supply. As the geographic location of the Tentsmuir site may have resulted in an increased proportion of 'older' cyprids throughout the experiments, a repeat of the experiment at an alternative location may provide an additional insight into *S. balanoides* behaviour.

Although the results indicated that *S. balanoides* cyprids settle preferentially on conspecific SF, significant levels of settlement occurred on allospecific extracts. However, *S. balanoides* settlement does not occur readily outside of the adult zonation (Hawkins, 1983). This suggests that other non-barnacle factors must also influence cyprid behaviour. In the laboratory, *S. balanoides* cyprids preferentially selected rock chips taken from the mid-shore, the natural zonation of the species (Thompson et al., 1998). This suggests that the natural biofilm, or a component from it may be influential. Strathmann et al. (1981) observed that *B. cariosus* settled preferentially on plates with biota characteristics of the adult zonation, concluding that an element of the microflora provided a settlement cue. Olivier et al. (2000) found that the microbial density, occurring as a result of emersion periods at different tidal heights, influenced *B. amphitrite* settlement. The drying of biofilmed surfaces affected the settlement behaviour of *Pomatoceros lamarkii* (Serpulidae) (Hamer et al., 2001). The authors were unable to determine the inhibitory effect of drying on the biofilm, though in the natural environment drying is linked to the period of immersion and therefore zonation. Similarly, a secondary biofilm cue may be important to *S. balanoides* settlement and may help to concentrate settlement in the mid shore. Additionally, cyprid pre-settlement distribution in the water column may influence settlement zonation (Miron et al., 1995 and 1999; Olivier et al., 2000)

**The unidentified species**

The cyprids of the additional species resembled *B. crenatus* (C.Todd pers. comm.). Additionally, *B. crenatus* adults are common along this coastline and, for example, are

abundant at spring low water at St Andrews Bay. However, the species was highly discriminating and appeared unaffected by the dominance of *S. balanoides* in the water column. The consistent significantly-greater settlement on *B. improvisus* SF, while the response to *B. crenatus* SF was no different to that of the other species, suggests that the cyprids were *B. improvisus*. While *B. crenatus* is most closely related to *B. improvisus* and also shares the same shore zonation, the conspecific cue should be preferred. If the cyprids were *B. crenatus*, interspecific pressures due to the mass of *S. balanoides* may have altered behaviour. However, this seems unlikely, as a significant settlement preference for *B. improvisus* SF continued during the late settlement period, when *S. balanoides* supply was much in decline. Alternatively, the SIPC content of the *B. crenatus* SF may have been limited, such that the signal from *B. improvisus* SF, a species of high systematic affinity, was favoured. However, the same preparation of SFs was used in all laboratory and field experiments, without similar effect. Irrespective of the identity of the species, it demonstrated a significant preference for the *B. improvisus* SF, with an equal response to all other SFs. Thus, the species was highly discriminating, and was seen to be more so than *S. balanoides*. While this may be inherent to the species, other factors, such as cyprid age and reduced intraspecific competition, cannot be ruled out. As settlement was dispersed across the slates with only one or two individuals per well (pers.obs.), intraspecific larva-larva reactions were considered less influential than the detection of a suitable adult cue.

The behaviour of the unidentified species was uniform throughout; it was not influenced by either intraspecific or interspecific interactions (due to the dominance of *S. balanoides*). In comparison, *S. balanoides* behaviour may have been influenced by intraspecific competition during the peak settlement (though a high cyprid density may have lead to deferment of settlement by individuals and a greater proportion of 'older' cyprids in the water column). Similarly, there was no evidence that interspecific interactions were influential.

### 2004 *Elminius modestus* trial

The Exmouth site had the advantage of being a virtual *E. modestus* monoculture, such that cues of other barnacle species that could influence behaviour were limited. A disadvantage was that the timing of the experiment was restricted by access to the site (i.e. for a few days during spring tides). The fundamental cause of the low settlement by *E. modestus* cyprids during the experiment was difficult to determine. The experiments were undertaken during the main settlement period. The presence of spat and juveniles already on *Mytilus* valves suggested that the timing was appropriate, though the period of peak settlement was not determined. The value of recording larval supply (Todd, 2003) was recognised. Juveniles were of varying size suggesting that settlement at the time of the experiment had occurred steadily over a protracted period, as would be expected for the species. While *E. modestus* may be the dominant barnacle larva in the water column during the summer months (Knight-Jones and Waugh, 1949), the associated continual release of nauplii results in larvae at different stages and an anticipated more-controlled supply of cyprids than that of *S. balanoides*.

Although low settlement may have mirrored low cyprid supply, other factors may have contributed. Slate has been used effectively in *E. modestus* settlement experiments (e.g. Larman and Gabbott, 1975; Barnett and Crisp, 1979), but the effectiveness of the current slate design has not been tested and the well shape may not have been optimal. Alternatively, the experimental panels were surrounded by conspecifics attached to *M. edulis*, perhaps representing a relatively attractive substratum compared to the slate. Laboratory experiments by Larman and Gabbott (1975) showed that *E. modestus* settlement was induced by extracts of *M. edulis*, at a level similar to that of the conspecific extract. The presence of conspecifics and a possible *Mytilus* cue in the immediate environment may have been sufficient for cyprids to preferentially select the natural habitat rather than the slate panels.

The observed lack of discrimination by *E. modestus* between different species accords with the findings of Knight-Jones and Moyse (1961) and Larman and Gabbott (1975), but contradicts the findings of other experiments (Barnett et al, 1979; Barnett and Crisp,

1979). The low level of settlement may have resulted in a biased outcome. However, settlement by the unidentified species during Experiment SBF3.1 was unambiguous despite low settlement. Thus, low settlement per se was not considered deleterious to the result. The age of the cyprids was an unknown factor and if the proportion of 'older' cyprids was high, then discrimination may have been affected, although laboratory experiments (Chapter 6) indicated that *E. modestus* discrimination to con- and allo-specific cues did not alter with age. The field result was the same as comparative laboratory experiments and the observed behaviour may be typical of what occurs in the natural environment. The lack of discrimination by this species, and in particular the lack of a secondary preference for the *S. balanoides* cue, suggested that the recognition of SF by *E. modestus* cyprids was different to that of *S. balanoides* cyprids. It suggests that *E. modestus* individuals either recognise an element of SIPC common to all species, or that their SIPC receptor is more broadly tuned than other species. The behaviour may have contributed to its success as a fouling species, and enabled its rapid invasion of UK and other waters. Further experiments at a different site and/or at a different time in the annual cycle would help to ascertain if the low rate of settlement and the lack of discrimination between species was typical. While it may be difficult to locate a further *E. modestus* monoculture, the emphasis would be to select a site with more moderate tidal forces and also with a lesser dominance by the species *M. edulis*.

## 7.4.2 Nitrocellulose membrane trials

### Suitability in the natural environment

The use of nitrocellulose membranes in field experiments would alleviate variation due to subtle substrate differences and the quantity of SF applied to treated areas. The seaward and landward positioning at Tentsmuir was selected to further test durability following the 2003 trial. The material remained intact and was considered durable, although the shore was relatively sheltered by the presence of the sandbar ca. 300 m from the coastline. When the membrane was used at Exmouth during the *E. modestus* trial, the strong tidal currents of the estuary were sufficient to destroy the material. The

use of nitrocellulose membrane in more extensive experimentation may thus be limited to sheltered environments.

### *Semibalanus balanoides* trial results

Gregarious settlement by *S. balanoides* cyprids was demonstrated clearly. While the level of settlement varied between assays, all results showed significantly higher settlement on conspecific-treated spots. For the final assay with BSA used as a control, the lack of control settlement suggested that larvae were able to distinguish clearly between the proteins. Settlement overall was low for this final assay and it may have been indicative of an inhibitory effect brought about by the presence of BSA, as observed in the laboratory (trial unreported experiments). However, the larval supply was reduced by the time of this assay, which was likely to have been a contributory factor. It would be worthwhile to repeat the BSA control assay earlier in the season. Additionally, following the success of the trial, the assay may be useful to further investigate con- and allo-specific settlement behaviour in the field.

## 7.5 Conclusion

Results of the 2002 experimental trial with the species *S. balanoides* indicated that the slates were both durable and suitable for experiments in the natural environment. The statistical analyses of the trial were not conclusive, but as settlement in control wells was not affected by adjacent treated wells, the method was considered robust. Settlement to *S. balanoides* and *E. modestus* SFs was ambiguous when analysed statistically and may have been influenced by limited replication. The result was used to inform the level replication for full scale experiments. The 2003 *S. balanoides* results were inconclusive, due to a reduced larval supply at the time of the experiments. However, the results indicated that *S. balanoides* larvae discriminated between species at settlement. The durability of the nitrocellulose membrane assay in field experiments was partially proven.

The 2004 results were more informative. *S. balanoides*, *E. modestus* and the unidentified species discriminated between SF and control treatments. However, discrimination between different SFs by *E. modestus* was low:-

(*E. modestus* = *B. crenatus* = *C. montagui* = *S. balanoides* = *B. improvisus*);

by *S. balanoides* moderate:-

*S. balanoides* > *E. modestus*> (*B. improvisus* = *B. crenatus* = *C. montagui*);

and by the unidentified species high :-

*B. improvisus* > (*B. crenatus* = *C. montagui* = *E. modestus* = *S. balanoides*);

where '>' indicates a significant preference and '=' indicates no significant difference.

*S. balanoides* settlement preferences varied between the 'early', 'mid' and 'late' assay periods and differences in behaviour during the mid-season may have been linked to an increased larval supply. The preference of *S. balanoides* cyprids for the *E. modestus* cue, over that of the more closely related *B. crenatus* and *B. improvisus*, suggested that conservation of an element of SIPC was more influential on settlement than systematic affinity. As cyprids settled to all cues, the influence of systematic affinity cannot be excluded. Preferential settlement by the unidentified species to *B. improvisus* SF suggested that cyprids were *B. improvisus* though a positive identification was not achieved. *E. modestus* settlement was low during the trial, though low settlement per se was not considered deleterious to the result. The result was comparable to laboratory findings. The recognition of SF by *E. modestus* was considered to be different to *S. balanoides* and may be typical of fouling species. Further experimentation may clarify species relationships.

Nitrocellulose was a durable substrate at Tentsmuir, though it was destroyed by fast-flowing currents at Exmouth and its use may be limited. The results obtained with *S. balanoides* cyprids at Tentsmuir suggested that the method may be useful in con- and allo-specific field experiments at sheltered locations.

# Chapter 8

# The conclusion

## 8.1 Introduction

The project aimed to investigate the utility of selected temperate barnacles as a model species for recently-developed laboratory assays, and to progress the understanding of barnacle con- and allo-specific settlement behaviour through laboratory and field experiments, and the partial characterisation of the adult cue. This chapter draws together the research outcomes and discusses them in the wider context of barnacle ecology and adaptation of species. Additionally the potential for further research is considered.

## 8.2 Summary of findings

Adult culture of *E. modestus* was achieved in small aquaria (semi-static culture) at a room temperature of 20±1°C on a 16:8 L: D cycle and fed daily on newly-hatched *Artemia* sp. The adults produced larvae continuously in these conditions. *E. modestus* larval settlement assays using a 24-well plate assay indicated a preferred settlement temperature of 22±1°C, and significant gregarious settlement was demonstrated at a SF concentration of 10 $\mu$g ml$^{-1}$. Experiments on the effects of ageing revealed a pattern to settlement not previously published for barnacles. Settlement was high when cyprids were young. It was reduced for middle-aged but then increased again with increasing age with an accompanying loss of discrimination to settlement cues. It was questioned whether the pattern resulted from specific physiology, whereby a single larval type exhibited different settlement behaviour at different ages, or whether the presence of different phenotypes, which either reached settlement competence at different times, or responded to different environmental cues, caused the observed pattern.

SIPC was isolated from *E. modestus* and two short amino acid sequences were obtained after several attempts. The genetic sequence was not achieved. Longer amino acid sequences would improve the chance of success, and it was recommended that the

protein isolation and amino acid sequencing should be repeated. Additionally, in view of the difficulties with digestion of the protein subunit, it may be appropriate to try an alternative species.

Laboratory experiments, investigating con- and allo-specific settlement behaviour of *E. modestus* and *B. amphitrite* larvae showed that there was no significant difference in settlement by cyprids to different species SFs. Additionally, field experiments identified settlement preferences for *S. balanoides* and a second unidentified species, while *E. modestus* larvae did not discriminate between different SFs. Overall, settlement preferences observed in the field experiments were defined as follows:-

*S. balanoides*:

*S. balanoides* > *E. modestus* > (*B. improvisus* = *B. crenatus* = *C. montagui*)

The unidentified species:

*B. improvisus* > (*B. crenatus* = *C. montagui* = *E. modestus* = *S. balanoides*).

*E. modestus*:

(*E. modestus* = *B. crenatus* = *B. improvisus* = *C. montagui* = *S. balanoides*)

where '>' indicates a significant preference and '=' indicates no significant difference. In laboratory and field experiments, settlement of all species was significantly greater on each SF tested compared to that on the control indicating that larvae were discriminating throughout.

## 8.3 Research findings in the context of barnacle ecology and adaptation

### Settlement preferences in relation to species development

The differences in larval settlement preferences are considered within the context of barnacle development. All barnacle species grow rapidly in early life and slower in later life, while large species tend to grow at slower rates and live longer than small species (Anderson, 1994). *E. modestus*, a species of relatively small adult size, is short-lived,

which has enabled it to make use of transitory available spaces (Foster, 1987). Individuals reach sexual maturity 38 – 50 days after settlement (Crisp and Bourget, 1985) producing multiple broods annually throughout their lifespan. Settled larvae will tolerate crowding as high as 230 individuals $cm^{-2}$ and in the race to develop will settle on and grow over their neighbours (Knight-Jones, 1952). The lack of discrimination between different species' SFs by *E. modestus* cyprids complements its opportunist life history. It is an additional factor that may explain its success as a fouling and invasive species. The life history of *B. amphitrite* is comparable to *E. modestus* and is a further example that supports a link between settlement preferences and life history.

In contrast, *S.balanoides* barnacles are annual brooders that are slow to develop. Sexual maturity is attained generally after 1 year of age and is often delayed until 2 years old (Barnes and Barnes, 1954). They are relatively long-lived; at mid shore they may live until after spawning in their third year, while further up the shore their lifespan may reach 5 – 6 years or longer (Moore, 1934). They favour less dense settlement than *E. modestus*. Knight-Jones and Moyse (1961) determined that individuals will settle at a density of up to 25 individuals $cm^{-2}$ if space is limited. However, at a density of 13 settled larvae $cm^{-2}$ an area becomes unfavourable for new individuals and is avoided if there is a bare space nearby. *S. balanoides* avoids settling on top of its own species, and in crowded situations, growth at the basal margin is restricted, but growth in height continues (Anderson, 1994). Thus, individuals tolerate their neighbours rather than smother them. The species is less opportunistic than *E. modestus* and has a greater need to settle in a community of conspecifics. The increased discriminatory ability of the species may be a contributing factor to the current success of this species.

**Survival in a changing world**

The potential impacts of global change are becoming of increasing concern and may affect both terrestrial and aquatic environments. Changes in ocean temperature, currents and salinity cause variation in oceanic biological populations (IGBP, 2003). Heat content of the oceans has increased over recent decades and current climate change models predict that global mean temperatures will rise by 1.4 – 5.8°C by the end of the

21st century (DEFRA, 2005). Theory suggests that the distribution of cold water species would shrink and move polewards, while that of warm water species would expand (IGBP, 2003). An increase in mean sea temperature of 2°C could result in latitudinal shifts of 200-400 miles in plankton distributions and result in extensive restructuring of marine communities (Southward et al., 1995). Tropical ocean waters have increased in salinity over the past 40 years, while oceans closer to the Earth's poles have reduced (Curry et al., 2003). By the end of the 21st century the climate is expected to be windier. Mean wave height is likely to increase, as will the frequency of storms. Predicted sea-level rises will shift the zonation of rocky shores landwards with the potential that the upper shore will be restricted (Kendall et al., 2004).

All species are likely to be affected. While many barnacles are euryhaline osmoconformers (Foster, 1970; Davenport, 1976) and should adapt easily to changes in salinity, other changes may result in new settlement distributions by many species. *E. modestus* is now absent from previously-colonised Portuguese coasts (O'Riordan and Ramsay, 1999), while new populations have been found in the Shetland Isles (Moore et al., 1995). Additionally, the warm water species, *Solidobalanus fallax*, common on the African coast, has recently colonised the English Channel (Southward 1995). Observations of *B. amphitrite*, *S. balanoides*, *E. modestus* and the unidentified species have shown that they all settle in response to con- and allo-specific cues, and other research has shown that these and other species behave similarly (Knight-Jones and Moyse, 1961; Larman and Gabbott, 1975; Barnett et al., 1979; Barnett and Crisp, 1979; Crisp, 1990; Whillis et al., 1990; Matsumura et al., 2000; Kato-Yoshinaga et al., 2000). Settlement in response to allospecific cues may assist in the survival of barnacles by enabling species to more easily colonise new geographic areas. Delayed metamorphosis should be minimised with individuals having good post-settlement growth potential. Such individuals may have a competitive advantage over other species in a changing community structure.

## 8.4 Further research

Further research into the following areas may extend the understanding of barnacle settlement behaviour.

### Laboratory experiments

#### *E. modestus*

A further investigation into the effects of ageing on *E. modestus* cyprids may improve understanding of the species' settlement behaviour. The development of a 'choice' assay that could test multiple cues may help to determine if more than one larval type is present in a single cohort. Additionally, a further investigation of IBMX induced settlement, using a greater range of concentrations, may improve the understanding of species' differences in the pheromonal modulation of barnacle settlement. An initial next step would be to test lower concentrations of IBMX, and to explore the efficacy of other chemical stimulants (see Clare et al., 1995). The nitrocellulose membrane assay had the potential to test different species cues simultaneously. However, consideration needs to be given to the possibility that cypris behaviour may have been influenced by the small closed environment of the assay container. The development of an alternative 'choice' assay to further investigate con- and allo-specific settlement behaviour in the laboratory might be necessary. Additionally, the use of purified SIPC in assays would alleviate the concern of variation in extracts between species.

#### *S. balanoides*

A further laboratory attempt to culture larvae would be worthwhile and it may be appropriate to incubate at 19°C with a controlled 16:8 L: D cycle and water changes every five days. If a successful larval culture was achieved, it would then be appropriate to consider adult culture and to attempt to extend the period of larval release. While the 24-well plate assay method was not successful, a further investigation of the nitrocellulose assays could be worthwhile.

## Field experiments

### *S. balanoides*

A further field investigation of *S. balanoides* settlement behaviour at an alternative site to Tentsmuir would be useful. It would be appropriate to select a site where the larval supply had not been prevented from settling due to an inappropriate substratum, such that the effects of ageing would be less equivocal. The nitrocellulose membrane assay trial could be extended for con- and allo-specific settlement and it would be useful to test BSA as a control with conspecific SF earlier in season.

### *E. modestus*

A further field investigation of *E. modestus* settlement behaviour at an alternative site, where there is less dominance of *M. edulis* and lower hydrodynamic forces, would not only provide a useful comparison to the results obtained at Exmouth, but would also enable the suitability of the experimental design to be determined. Additionally, it would be appropriate to reattempt the nitrocellulose membrane trial with *E. modestus*.

### St Andrews Bay area

It would be valuable to identify the unidentified species by growing on individuals and consider further con- and allo-specific experiments with this species.

## SIPC-focused research

A further attempt to isolate and identify SIPC of a second species is essential to progress the understanding of gregarious settlement at a molecular level. If difficulties continue with the digestion of the *E. modestus* isolate, the use of an alternative species may be appropriate and *S. balanoides* could be used. More detailed investigations of SIPC in the context of barnacle physiology, as identified in Point 5 of Chapter 1 Section 1.7.2 would also progress understanding.

## 8.5 Conclusion

The aims of this research project were to identify a temperate species suitable for use as a model organism, to test con- and allo-specific settlement on SF in laboratory and field situations and to isolate SIPC from a second species. The aims have been achieved, though there are many questions still to answer. It is more than 50 years since gregarious settlement behaviour was first identified in barnacles and the quest to fully understand this behaviour has many facets still to be investigated.

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