THE UNIVERSITY OF HULL

# MATE CHOICE IN THE POLYCHAETE *NEREIS ACUMINATA*: THE ROLE OF AGGRESSION AND PARENTAL EXPERIENCE.

being a thesis submitted for the Degree of Ph.D.
in the University of Hull

by

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# Acknowledgements

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## Abstract

*Nereis acuminata* is a polychaete species with a unique life cycle. A male and female form a monogamous pair bond, the female lays eggs and dies. The male then fertilises the egg and undertakes sole parental care until the eggs hatch and larvae leave the parental burrow; the male can then reproduce again. This body of work investigated aggression, pair formation, filial cannibalism, the scent of experience and the occurrence of male parental care for eggs fertilised by another male in the polychaete species *Nereis acuminata*.

Female aggression following egg release was found to be absent when the females were not in the presence of an egg mass, with no aggression displayed towards sexually mature females. Aggression between two males did not have a subsequent effect on the pairing behaviour with a female, although there was slight decrease (not significant) in the time taken for individuals to pair if they had previously formed a pair bond. Males that had previously had an aggressive encounter were fought subsequently to determine if there was recognition for previous opponent but aggression did not change following previous fights, indicating that previous fights between the same opponents does not have an effect on the aggression levels exhibited. Aggression between the populations maintained in the laboratory, Reish (R), Newport (N), San Gabriel (SG), Connecticut (C) and the wild population Los Angeles (LA) were examined. Although there were significant differences in male aggression between the R, N, SG and C populations, and significant differences in female aggression between the R and LA populations, aggressive behaviour was not found to be a strong indicator of population divergence. Observations of pair formation, however, provided a stronger indicator of divergence. Pairing behaviour within each population was found to be significantly different to that between individuals from different populations, with Connecticut individuals failing in the majority of cases to form a pair bond with any of the other three laboratory populations. The LA and R populations, sampled in the same location 44 years apart, did not form pairs with the same frequency as males and females from the same population but interactions between R and LA males and females were found to form a high frequency of pairs, indicating that although the R population may be heavily inbred, females of this population will still form monogamous pairs with males from the LA population. The behavioural and molecular evidence suggests that these populations form a species complex and this is important to note when undertaking

ecotoxicology testing as the different populations may respond differently to the same environmental conditions.

The occurrence of filial cannibalism was investigated in male *N. acuminata*, looking at aggressive interactions between males and female mate choice. The presence of a cannibalistic male did not affect the aggression observed between two individuals and it appeared that females could not discriminate between males that had cannibalised their egg mass. Conditioned water was used from various sources to determine if female preference could be altered following an initial selection of male by the female. Water from males caring for eggs and from new experienced males that had just completed egg care was found to change the choice of the female to a pre-treatment loser. Water conditioned from females, juveniles and inexperienced males appeared to enforce the previous choice of the female as in these trials the female chose the previous winner more frequently. The mechanisms related to the scent of experience in terms of physiology and release of the chemical signal however, are still unknown. It is thought that there is a physiological change in the male, for example by release of sperm or by production of ‘new’ sperm that the female can detect and uses to indicate that a male has previously cared for a brood. As the female dies following egg release and the male undertakes sole parental care, an honest indication of the ability of the male to successfully care for eggs is vitally important.

Finally, male behaviour towards an egg mass fertilised by another male was investigated. Inexperienced males that had not completed egg care were found to care for the eggs until they hatched whereas experienced males were not, instead cannibalising the egg mass. Inexperienced males were likely to gain from adopting eggs by gaining parental experience and receiving more matings in the future. Experienced males did not gain benefits from caring for unrelated eggs but cannibalising the eggs would benefit the experienced male by providing extra nutrients.

*Nereis acuminata* is an ideal species to use as a model organism to investigate the behavioural and evolutionary processes involved in mate choice, aggression, preference for parental experience and the chemical signalling involved, due to its adaptable life history, short life cycle and ease of maintenance in the laboratory.

# <u>Chapter One:</u>

# Literature Review

### 1.1: Research rationale

The life cycle of *Nereis acuminata* is unique among nereid polychaetes (Bridges *et al*., 1996) with female death following reproduction by dehiscence (the splitting of the body wall); sole male parental care of eggs and the ability for the male to reproduce again once egg care is complete (Reish, 1957; Reish, 1985). Although the life cycle of *N. acuminata* has not been observed in the field, this species can still be used as a model for evolution as it has an easily adaptable life history, a relatively short life cycle and is convenient to use for behavioural bioassays with observations easy to make and experiments simple to conduct at each stage of the life cycle (Reish, 1980b; Reish, 1985).

Populations of *N. acuminata* have been sampled worldwide but the taxonomy of this species is still unclear. Populations may in fact form distinct, reproductively isolated units depending on their location. The chromosome numbers of some of the populations of *N. acuminata* have been identified. The diploid chromosome number of the Pacific Ocean populations (California, U.S.A) is 18 (Pesch and Pesch, 1980; Weinberg *et al*., 1990). The diploid chromosome number of the Atlantic populations (Connecticut, U.S.A.) is 22 (Weinberg *et al*., 1990). Differences in chromosome number can be an indication of reproductive incompatibility (Knowlton, 1993). Although populations examined share the same life history traits, previous studies have indicated that separate populations behave differently, for example in pairing experiments between populations (Weinberg *et al*., 1990; Weinberg *et* al., 1992; Sutton *et al*., 2005). As *N. acuminata* is used in ecotoxicology studies to test for water quality and the presence of heavy metals bound to the sediment, differences in population behaviour may have wider implications; different populations may behave differently when used in such tests, for example in terms of metabolism, growth or reproduction. Differences in pairing behaviour between the populations has led researchers to suggest that *N. acuminata* populations may be reproductively isolated (Weinberg *et* al., 1990; Weinberg *et al*., 1992; Sutton *et al*., 2005); known as the final step before speciation (Snell, 1989). Populations of this species may therefore form a species complex if reproductive isolation occurs.

Although sexual selection has been examined in *N. acuminata* (Starczak, 1984; Fletcher, 2004, Storey, 2006, Fletcher *et al*., 2009), the process is still not fully understood. As the female dies following reproduction, it is vital that the female makes a good mate decision as the reproductive success of her offspring depends entirely on the ability of the male to care for offspring. Although males undertake sole parental care, female choice for males that have previously fathered a brood has been shown to occur (Fletcher, 2004, Storey, 2006, Fletcher *et al*., 2009) and female reproductive input is higher than the input of males in this species due to the occurrence of female death following reproduction (Starczak, 1984). The behaviour of males prior to reproduction with a female may have an effect on female choice. Both cannibalism and adoption of eggs have been reported to occur in *N. acuminata* (Oshida *et al*., 1981, Sutton, 1998) but the full effects of these forms of behaviour in terms of subsequent female mate choice have not been expanded upon. Although female mate choice has been shown to occur, the mechanism by which this advantage is conferred to females is still unclear.

This thesis attempted to address the above questions and problems. The more we know about the life history stages in this polychaete and the interactions between the populations, the more we can apply that knowledge to use *N. acuminata* as a model species not only as an indicator of pollution and water quality monitoring and population divergence, but also as model for sexual selection theory.

## 1.2: Annelid polychaetes

Polychaetes are thought to represent 40% of all the species constituting the soft bottom sub-tidal benthos (Reish, 1980b) with approximately 8,000-9,000 different species (McHugh and Fong, 2002; Ruppert *et al.*, 2004). The general anatomy of *Nereis spp.* consists of a linear array of segments, with each segment acting as a sheath for the coelom (Kershaw, 1983). The body consists of a prostomium and peristomium which form the head region, and also a pygidium, which forms the tail region and bears the anus (Knox, 1951). In the head region, there is an eversible pharynx which is used to capture food (Kershaw, 1983). The pharynx has a single pair of jaws with serrated edges (Knox, 1951), controlled by muscles in the wall of the proboscis (Kershaw, 1983). The circulatory system is closed and blood flows to the head region in a dorsal vessel and away from the head in a ventral vessel by peristaltic action (Flint, 1965). Gas exchange can occur across the cutaneous surface of the epidermis but some polychaetes have gill structures, which are often a modification of one set of parapodia (Withers, 1992). The brain of nereid polychaetes is bi-lobed and the control of endocrine function is well-developed (Withers, 1992). Individuals are also able to respond to tactile and chemosensory stimuli. The epidermis houses the sensory nerve cells and the sense organs (Dyal, 1973). The anterior region of the body usually contains sensory organs such as the palps, cirri, antennae, proboscis and eyes (Flint, 1965). The chemoreceptors are located in nuchal organs found at the junction between the prostomium and peristomium (Dyal, 1973). Digestion enzymes are excreted from the epithelial lining via the nephridia as food passes through the continuous gut. The products of digestion are then absorbed in the gut wall by blood capillaries (Kershaw, 1983). Sperm and egg maturation occurs mainly in the coelomic fluid (Rouse and Pleijel, 2006). When gametes are ripe, they pass to the exterior of the body through the segmental organs known as coelomoducts but in some species the eggs are released by dehiscence (Borradaile *et al.*, 1963). Movement of the body via contraction of the longitudinal muscles is coordinated by the giant axon of the ventral cord (Horridge, 1959) in response to the excitation of the supra- and sub-pharyngeal ganglia (Borradaile *et al.*, 1963).

The experimental species *Nereis acuminata*, like most nereids, has four eyes, two antennae, two palps and four pairs of dorsal and ventral tentacular cirri. The proboscis is strong and eversible with oral and maxillary rings, terminated with horny falcate jaws (Moore, 1903; Pettibone, 1963). Further anatomical detail can be seen in Figure 1.1 below.

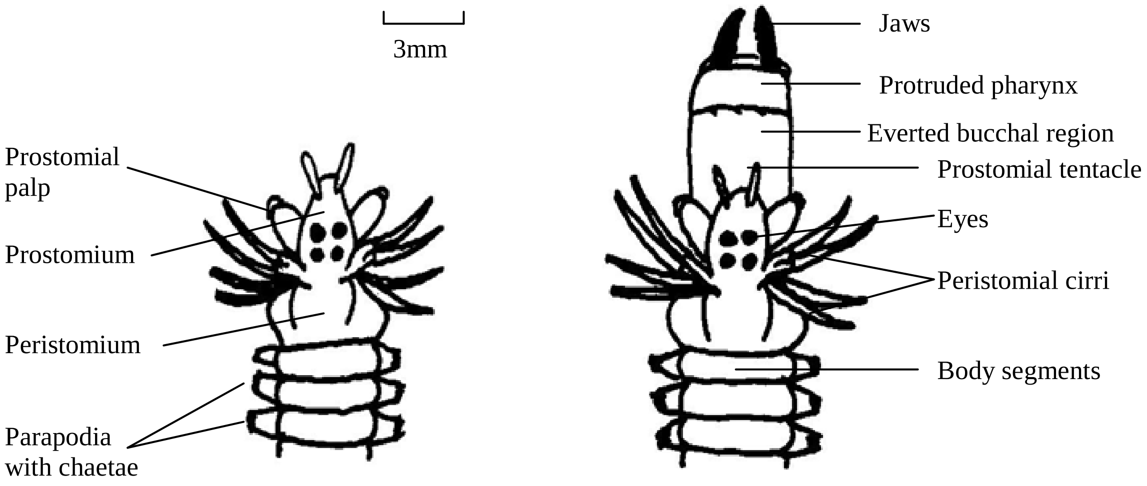


Figure 1.1: Dorsal view of the head region of a typical *Nereis acuminata* individual, with everted pharynx on the right; adapted from Bhamrah and Juneja (2001).

### 1.3: The experimental species and previous research

The polychaete species, *Nereis (Neanthes) acuminata* (Ehlers, 1868 cited Kudenov, 1975) is also known by six other names including *Nereis (Neanthes) caudata* (delle Chiaje, 1828), *Spio caudatus* (delle Chiaje, 1828), *Nereis (Neanthes) arenaceodentata* (Moore, 1903) *Nereis (Neanthes) cricognatha* (Ehlers, 1904 cited Knox, 1951) *Nereis (Neanthes) eikini* (Hartman, 1936 cited Pettibone, 1963) and *Nereis (Neanthes) bolivari* (Rioja, 1918 cited Barnich *et al*., 2000). There appears to be considerable confusion in the literature regarding the naming of the experimental species and the taxonomy is unclear (Pettibone, 1963). *Nereis* has been described as an old name for *Neanthes* by Bhamrah and Juneja (2001) but *Neanthes* and *Nereis* are described as possessing chaetae that are different in their morphologies (Muir and Bamber, 2008). At the end of the chaetae, the setae (bristles) have a shaft with a terminal end composed of two branches. If these branches are the same length they are described as homogomph; if they are of different lengths, they are described as heterogomph. The terminal end of each of the branches can either be long and pointed, described as a spiniger, or short and hooked described as a falciger (Knox, 1951). *Nereis* is described as a genus with individuals possessing homogomph spinigers and falcigers in the middle and posterior notopods whereas individuals of the genus *Neanthes* have homogomph spinigers in the notopods only (Knox, 1951). In the literature, however, *Nereis* and *Neanthes* appear to be used interchangeably for this species and there is no distinction made between the morphologies of each genus. A summary of the different pseudonyms is detailed in Table 1.1. In the literature, Day (1973 cited Reish, 1985) appears to have dismissed the use of *Nereis (Neanthes) arenaceodentata* in favour of *Nereis (Neanthes) acuminata* (the earliest name used) and this was subsequently adopted by Gardiner (1975), Taylor (1984 cited Moore and Dillon, 1992) and Weinberg *et al*. (1990). However, *Neanthes arenaceodentata* is the most commonly used pseudonym in the ecotoxicology literature where most of the specimens are listed as originating from a population held in the laboratory of Dr. D.J. Reish, sampled from California on the west coast of North America. However, Bakken (personal communication) states that *Neanthes arenaceodentata* is an east coast population, originally sampled in New England. The species described here all have a similar morphology and life history traits and it is hypothesised that they may form part of a species complex composed of sibling species (Weinberg *et al*., 1990). Sibling species are populations that are morphologically similar or identical that are reproductively isolated (Mayr, 1977). In the case of this body of work, for ease of description, the species used will be referred to as *Nereis acuminata* (henceforth *N. acuminata*).

Populations of *N. acuminata* which share the same life history traits and morphology have been reported in various coastal locations across the world as demonstrated in the distribution map on the following page in Figure 1.2. The numbers used for each pseudonym in Table 1.1 above are also represented on the distribution map to show approximate geographical regions for each pseudonym. For example, the species sampled along the east coast of North America is described as *Neanthes acuminata* whereas the species sampled in the Caribbean is described as *Neanthes arenaceodentata*. This further highlights the confusion concerning the taxonomy of this species as the names given to the populations sampled in each geographical location vary considerably. It is still unknown whether each of these populations should be classed as separate species.

Table 1.1: The original sampling locations for the experimental species, including the different nomenclature used.

| Name | Location | Reference |
|---|---|---|
| 1. *Nereis acuminata* | Mediterranean | Ehlers, 1868 cited Kudenov, 1975. |
| 2. *Neanthes acuminata* | East coast USA, | Ehlers, 1868 cited Kudenov, 1975. |
| 3. *Nereis caudata* | Mediterranean, Gulf of Naples, Egypt | delle Chiaje, 1828. |
| 4. *Neanthes caudata* | Mediterranean, Egypt | delle Chiaje, 1827 cited Pettibone, 1963 |
| 5. *Nereis arenaceodentata* | Caribbean | Moore, 1903. |
| 6. *Neanthes arenaceodentata* | West coast USA | Moore, 1903. |
| 7. *Nereis eakini* | Mexico, Peru, Galapagos Islands | Hartman, 1936 cited Méndez, 2006. |
| 8. *Nereis bolivari* | Spain | Rioja, 1918 cited Barnich *et al.*, 2000. |
| 9. *Nereis cricognatha* | New Zealand, Australia | Ehlers, 1904 cited Knox, 1951 |
| 10. *Spio caudatus* | Mediterranean | delle Chiaje, 1828. |

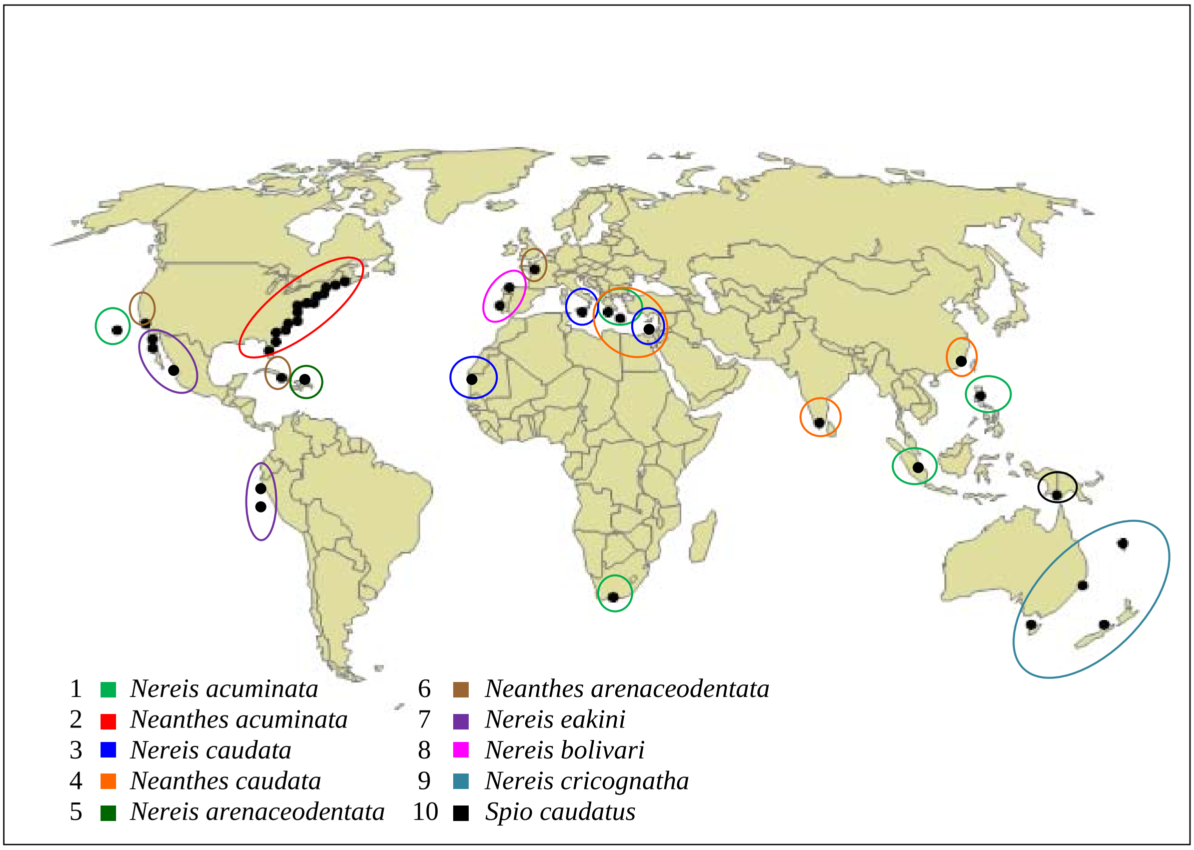


Figure 1.2: Distribution map of known sampling sites for *N. acuminata* (delle Chiaje, 1827 cited Pettibone, 1963; Ehlers, 1904 cited Knox, 1951; Rioja, 1918 cited Barnich *et al.*, 2000; delle Chiaje, 1828; Ehlers, 1868 cited Kudenov, 1975; Moore, 1903; Fauvel, 1923 cited Clark, 1959; Herpin, 1926 cited Reish, 1957; Hartman, 1936 cited Méndez, 2006; Reish, 1957; Pettibone, 1963; Reish, 1980a; Pesch *et al.*, 1987; Weinberg *et al.*, 1990; Wolff *et al.*, 1993; Muir and Bamber, 2008; Abd-Elnaby, 2009).

This species occupies mucus-lined burrows in the upper 2-3cm of brackish and marine sediments (Bridges *et al.*, 1996), mainly found at low water levels in sand, but also on mussel beds, sponges or on floating algae (Pettibone, 1963). Many species of polychaetes are known to construct mucous-lined burrows. These are often sticky, allowing the external surfaces of the burrow to attach to bare surfaces. Sand, debris, mud and shell fragments often become integrated and provide extra strength to the burrow. Such burrows provide the inhabitant with protection, aid in prey capture and in some species, provide a nest site for eggs and young (Ruppert *et al*., 2004). *N. acuminata* is able to extend from the burrow to feed without vacating it completely, presumably to avoid predation from fish and bird species which are their main predators (Hutchinson *et al*., 1995). Although *N. acuminata* are opportunistic omnivores (Reish, 1957), their typical diet consists mainly of diatoms and filamentous algae such as *Enteromorpha spp.* (Herpin, 1926 cited Reish, 1957). Individuals have been known to be carnivorous and are able to use their jaws to attack and digest prey larger than themselves (Herpin, 1926 cited Reish, 1957). The proboscis ends with two brown curved jaws, each with approximately ten teeth (Pettibone, 1963). Individuals typically grow to 70mm long, 40mm in width and have approximately 75 segments (Pettibone, 1963).

The life cycle of *N. acuminata* has not been documented in the field (Starczak, 1984). However, under laboratory conditions, *N. acuminata* individuals do not follow a distinct breeding season as constant ambient and water temperatures are maintained. Instead, this species reproduces all year round once sexual maturity is reached (Starczak, 1984). A complete life cycle documented under laboratory conditions can be seen on the next page in Table 1.2.

Table 1.2: Life history traits of *N. acuminata* (adapted from Reish, 1957).

| Age (days) | Characteristics | Approx. length (mm) |
|---|---|---|
| 7 | Egg elongation, muscular movement. | 0.52 |
| 8 | 3 larval segments, anal cirri, hatched from egg capsule. | 0.62 |
| 9 | 4 larval segments, 1 pair of peristomial tentacles. | 1.00 |
| 10 | 5 larval segments, 2 pairs of peristomial tentacles, early palpi, jaws with terminal tooth only. | 1.10 |
| 11 | 7 larval segments, 3 pairs of peristomial tentacles. | |
| 13 | 12 adult segments, seta of 1st larval segment drop out, segment becomes peristomium, jaws with 4 lateral teeth. | 1.40 |
| 14 | 14 segments, jaws with 5 lateral teeth, paragnaths on maxilliary ring only. | |
| 16 | 16 segments, jaws with 6 lateral teeth. | |
| 21 | 18 segments, jaws with 7 lateral teeth, jaws become dark brown at tips, paragnaths present on oral ring, vacate parental tube. | 4.00 |
| 23 | 24 segments, 4 pairs of peristomial tentacles, feeding. | 6.00 |
| 26 | 32 segments, paragnaths become dark brown. | |
| 33 | 42 segments. | |
| 36 | 48 segments. | |
| 40 | 51 segments. | 7.00 |
| 44 | 55 segments, eggs observed in female coelom. | 11.00 |
| 65 | Female lays eggs and dies, with male incubating. | 35.00-70.00 |
| 92 | Male fertilises eggs from second female. | |
| ~300 | Male dies. | |

Nereid polychaetes can either be referred to as iteroparous or semelparous, depending on the life history of the species. Iteroparous individuals can reproduce more than once per lifetime, either continuously or at short intervals (Prevedelli and Simonini, 2003). In contrast to this, in semelparous individuals, reproduction terminates the life cycle (Clark, 1965). Nereids can be further subdivided into those species that reproduce in an epitokous form and those that reproduce in an atokous form. Epitokous individuals undergo a metamorphosis into a special reproductive form, also known as a heteronereid (Clark, 1961). Atokous individuals however, do not undergo such a metamorphosis and there is no planktonic larval stage (Clark, 1961). Reproduction in nereids can occur via a mass spawning event, triggered by environmental and endocrine cues, maximising the fertilisation rate due to an increase in gamete interaction (Hardege and Bentley, 1997). However, mass spawning is not the only mode of reproduction found in nereids; internal and self-fertilisation also occurs, along with egg-laying.

Some examples of polychaetes that reproduce via a mass spawning event include *Nereis vexilosa*, *N. succinea* and *Platynereis dumerilii*. These species reproduce in an epitokous form and have a semelparous life history (Johnson, 1943; Beckmann *et al.*, 1995; Hardege *et al.*, 2004). *N. vexilosa* females release a mass of eggs which is followed by the male spawning directly into the water column (Johnson, 1943). In both *N. succinea* and *P. dumerilii*, individuals assemble near the water surface at night (Beckmann *et al.*, 1995) and spawning is synchronised by complex environmental and endocrine cues such as day length, lunar cycle and chemical cues (Hardege *et al.*, 2004). Complex bouquets of pheromones are used to coordinate both nuptial dance behaviour, where males swim round females in narrowing circles and spawning takes place (Clark, 1961) coordinate the release of gametes (Zeeck *et al.*, 1988). The pheromone cysteine-glutathione disulphide (CSSG), also termed “nereithione” is found in the coelomic cavity of both of these species and is released by females during reproduction, acting to aid in mate recognition (Ram *et al.*, 1999) and inducing sperm release in males (Zeeck *et al.*, 1998b). At low concentrations of CSSG, males follow trails to locate females; spawning is induced in males with high concentrations of CSSG (Ram *et al.*, 2008). *N. succinea* and *P. dumerilii* do not spawn at the same time not only because each species initiates spawning at a different time in the lunar cycle (Watson *et al.*, 2003), but also because the pheromone concentration required to elicit the reproductive response differs in each species (Hardege *et al.*, 1998). Reproductive behaviour in *P. dumerilii* was found to be further controlled by 5-methyl-3-heptanone (Beckmann *et al.*, 1995) and uric acid (Zeeck *et al.*, 1998a) and males of this species also produce an egg-release pheromone, composed of inosine and glutamic acid (1:1) (Hardege *et al*., 2004). Other polychaetes that reproduce via a mass spawning event include *N. virens* (Olive *et al*., 1997) and *N. japonica* (Smith, 1958).

The semelparous species *Platynereis megalops* has an unusual mode of reproduction as fertilisation is internal (Just, 1914). Sexually mature individuals swim at the water surface and the smaller males swim rapidly in spirals around the larger females in narrowing circles. The female becomes trapped in the coil as the male begins to straighten out. Once the female is trapped, the male inserts his tail into the jaws of the female where the eggs are packed within the coelomic cavity and fertilises the eggs with sperm. The male and female then separate and eggs emerge by dehiscence (Just, 1914). Fertilisation of eggs is internal, which is unique in nereid polychaetes (Smith, 1958) and eggs cannot become fertilised outside of the female (Just, 1914).

*Nereis limnicola* is a viviparous self-fertilising hermaphrodite (Baskin, 1970). Individuals reproduce in an atokous form (Smith, 1958) and parturition of larvae occurs through the body wall (Baskin, 1970). Gametes are fertilised in the coelom and development proceeds in the coelomic cavity until the larvae are roughly 4-5mm in length (Baskin and Golding, 1970). The coelomic milieu provides the developing larvae with essential nutrients and other components required for growth (Baskin and Golding, 1970) and also protects from osmotic shock (Evans, 2009). In *N. diversicolor*, when a male detects the presence of a ripe female, males release sperm in front of the female tube, with no direct contact between the two individuals. The female then transports sperm into the burrow using the proboscis (Bartels-Hardege and Zeeck, 1990) and eggs are released spontaneously by dehiscence (Smith, 1958).

In *N. acuminata*, the complete reproductive cycle is unique among nereid polychaetes (Bridges *et al*., 1996), although some reproductive characteristics are similar to those of other species. *N. acuminata* does not undergo epitoky to a heteronereid form (Reish, 1957). When a male and a female come into contact, there is a slowing of movement (Bridges *et al*., 1996), with the male and female forming a monogamous pair bond and constructing a new mucous-lined burrow (Reish, 1985). Burrows are composed from excretions from glands situated near the parapodia to form a collar that is slowly extended to form a tube around the individual (Daly, 1973; Pleijel and Dales, 1991). After the eggs (measuring up to 650µm) have been shed, the female vacates the shared burrow to die (Reish, 1980b). The male then fertilises the egg mass and is solely responsible for parental care, circulating water over the developing eggs using body undulations (Reish, 1980b). Male incubation of eggs has also been observed in *Platynereis massiliensis*, *Micronereis variegata*, *M. nanaimoensis*, *Laconereis glauca* and *Ceratonereis costae* (Reish, 1957). After egg care is complete, once juveniles exit the tube and take up active independent feeding, the male is then able to reproduce again, as in *P. megalops* (Just, 1914). Males of this species have been observed under laboratory conditions to reproduce and produce successful broods up to seven times (Reish *et al.*, 2009). In this species therefore, the female is classed as semelparous but the male is iteroparous (Reish, 1957). Emergent juveniles exhibit weak dispersal (Weinberg *et al.*, 1990), undergo direct development and therefore do not go through the planktonic trochophore larval stage, instead developing into benthic metatrochophore larvae, with segmented bodies and a distinct head (Moore and Dillon, 1992).

The theory of sexual selection predicts that mate choice will evolve when mating partners vary in genetic quality or with the ability to provide benefits to mating partners (Andersson, 1994). Selected mates can either provide direct benefits, which can include parental care quality, increased fertility (Searcy, 1982) nest sites, territories, nuptial gifts, absence of parasites (Møller and Thornhill, 1998), food for mates and/or predator defence (Yasui, 2001), or indirect benefits, such as genetic fitness (Yasui, 2001). It is likely that the gender with the lowest potential reproductive rate should be choosy (Kraak and Bakker, 1998) and the sex that provides the greater level of parental care investment should be more discriminating (Searcy, 1982). Added to this, a skewed Operational Sex Ratio (OSR), the ratio of males to females in a population that are ready to reproduce (Emlen and Oring, 1977), can lead to increased competition among members of the more abundant sex. Generally, females are choosy and males are more active in courtship, known as the ‘traditional’ male sex-role (Berglund *et al.*, 1986). Reproduction in females is expected to be more costly due to the limitations of egg production and the number of young they can produce. Sperm are generally less costly to produce than eggs and only a small amount of energy is invested in sperm production (Pitnick and Markow, 1994). Female choice and assessment of mates has therefore led to the evolution of multiple male traits and the preference of females for such traits (Kodric-Brown, 1995). Male traits can signal to a female that he is in good physical condition (Cotton *et al.*, 2006) and are often a sign of good genetic quality (Andersson, 1982). Such traits may include superior fighting ability, provision of breeding resources and/or the quality of male parental care (Pampoulie *et al.*, 2004). Females may not receive direct benefits from males and should therefore select for health and genetic quality and prefer traits that are an honest indicator of the heritable variation in male genetic quality (Hoelzer, 1989). Such males are less likely to carry disease and are more likely to have viable sperm (Fisher and Rosenthal, 2006).

Three models have therefore been proposed to explain mate choice in females for such traits in males: the Fisherian process; the good genes process; and, the good parent process. The Fisherian process proposes that females choose males to produce "sexy sons and choosy daughters". Traits that are selected for by the female will also be expressed in the offspring, for example if males with bright plumage are selected, sons will also have bright plumage and daughters will select a mate also based on bright plumage (Fisher, 1930 cited Zahavi *et al*., 1975). Once sexual selection begins to have an effect, the preference itself creates a selective pressure which leads to the exaggerated development of the trait (Zahavi *et al*., 1975). The good genes process hypothesises that a female may increase the fertility of her offspring by selecting a mate on the basis of good genes. If a female selects a mate with 'good genes', the female can improve the viability or fertility of offspring; such trait evolve because they are correlated with genetic quality of the potential mate (Zahavi *et al*., 1975). Finally, with the good parent process, females should select traits that advertise good male parental care ability, when such care influences the viability or fertility of offspring (Hoelzer, 1989).

Species with male mate choice are referred to as being sex-role reversed. This occurs when females compete to mate and males are choosy (Ridley, 1978). Sex-role reversal is expected to occur if mating is costly in males (Amundsen *et al*., 1997), if female quality varies, if there is a female-biased OSR, if the energy investment per female varies (Côte and Hunte, 1989), if females can re-mate faster than males (Berglund *et al*., 1992), if males have a lower reproductive rate than females (Svensson, 1988) or if males invest more in gametes than females (Gwynne, 1981), for example in the giant sperm species *Drosophila bifurca,* where gamete production in males is limited (Luck and Joly, 2005). In many sex-role reversed species, the male is the sole provider of parental care (Ridley, 1978) and male mate choice therefore correlates to the higher reproductive input of the male into his offspring (Berglund *et al*., 1986). Females may compete not just for access to mates, but also for mating resources such as food and territory (Bebié and McElligott, 2006).

Mutual mate choice occurs predominantly in monogamous species where individuals have similar reproductive rates and there are benefits to both sexes (Amundsen *et al*., 1997). In the redlip blenny *Ophioblennius atlanticus* for example, males and females prefer to mate with larger individuals. Females were found to release more eggs with larger males and in turn, larger males had a higher hatching success and were better at egg guarding. Larger males were also found to spend longer in the nest and had fewer egg losses than smaller males. Large females were found to be preferred by males due to the higher number of eggs produced by such females and greater egg numbers offset the associated costs of eggs guarding (Côte and Hunte, 1989).

Although male *N. acuminata* undertake sole parental care of eggs, such behaviour does not always indicate that a species is sex-role reversed (Vincent *et al*., 1992). Even though there are high levels of investment in offspring by both males and females of this species (Starczak, 1984), female reproduction costs due to female death following egg dehiscence are likely to be higher than costs incurred by males. Males are also able to reproduce again following completion of egg care (Reish, 1957), meaning males have a lower investment than females (Starczak, 1984). It is therefore unlikely that this species exhibits male mate choice. Males are also more likely to engage in parental care if the likelihood of paternity is high (Ridley, 1978), where eggs will not survive without the presence of a male to undertake oxygenation, cleaning and defence of eggs (Reish, 1957). As males defend females before the egg mass is laid, and the fertilisation event is external, fertilisation by the guarding male is ensured and he is therefore highly likely to be caring for eggs that are his own. Also, mating is non-random (Starczak, 1984) as female choice for males has been observed and studies indicate that choice is based on chemical cues received by the female (Starczak, 1984; Fletcher, 2004; Storey, 2006). Females of this species do exhibit a preference for dominant males but overall prefer experienced males, those that have previously produced a viable brood (Fletcher 2004; Storey, 2006, Fletcher *et al*., 2009). Berglund *et al*. (1986) has also shown that extensive male care does not necessarily lead to sex-role reversal. Therefore, it is indicated that female *N. acuminata* exhibit mate choice based on the good parent model of sexual selection, where males display to females the desired trait of completion of egg care and the successful rearing of a brood (Hoelzer, 1989). It is thought that this evolved due to the death of the female following egg release (Starczak, 1984). It has been shown that parental care in *N. acuminata* may increase future reproductive success (Fletcher *et al*., 2009).

In polychaetes, aggression involves eversion of the proboscis, thrust forward toward the opponent (Reish, 1957), with the jaws used for biting or grasping (Weinberg *et al*., 1990). Studies regarding aggressive behaviour in *N. acuminata* have shown that two individuals of the same sex will fight but also that a male will fight an intruder of either sex during parental care (Reish, 1957). Once egg care is complete, however, a male will no longer fight a female as he is able to reproduce again (Reish, 1957). Spent females (those that have laid eggs) are also known to fight with introduced females that have not yet laid eggs (Reish, 1957). Aggression was also observed to alter following male interactions with females; after pairing with a female, aggression between two males increased if they were returned to fight with each other. If a female was present however, aggression was observed to decrease (Fletcher, 2004). Aggressive encounters have been found to be more frequent at increased densities with larger individuals more successful at defending larger territories (Bridges *et al*., 1996).

During parental care, male *N. acuminata* have been observed to cannibalise eggs during incubation (filial cannibalism) (Reish, 1957). Cannibalism has also been observed to occur at other stages in the life cycle of this *N. acuminata*. Not only have individuals engaging in aggressive encounters been shown to be cannibalistic (Reish, 1985), but male *N. acuminata* are also known to consume the female once she has released her eggs (Reish, 1957). Two other known cannibalistic polychaetes are *Platynereis bicaniculata*, shown to attack and cannibalise burrow intruders (Woodin, 1974) and *Nereis grubei* which is cannibalistic when food resources are low (Reish and Alosi, 1968). In *N. acuminata*, as in many other marine invertebrates (Dreon *et al*., 2006), eggs are rich in lipovitellin that provides energy and nutrients to larvae when they first hatch (Lee *et al*., 2005). Filial cannibalism may therefore occur for a number of reasons: via accidental consumption (Schabetsberger *et al*., 1999), to clean the burrow/nest of dead and diseased eggs (Kraak, 1996) and may also provide the male with nutrients and energy to continue care of the brood until the larvae exit the tube and take up independent foraging (Polis, 1981). Eggs are an easily-attainable resource, rich in nutrients and are also defenceless (Acha *et al*., 2002), although eggs may show a form of defence against predators by containing toxins or being unpalatable, as found in the toad *Bufo valliceps* (Licht, 1968; 1969). Parental care may be costly in terms of reduced foraging opportunities (Lindström, 1998) so filial cannibalism may also function to offset the cost of such care (Okuda and Yanagisawa, 1996). Consuming eggs may not fully compensate for the cost of care (Kraak, 1996) but has been found to have the potential to increase future reproductive success by survivorship of the parent

(Lindström and Sargent, 1997). Filial cannibalism may also occur when the brood is smaller than normal as the reproductive value of the clutch would not outweigh the costs associated with care of that brood (FitzGerald, 1992).

As well as the studies conducted to further understand behaviour and life histories, polychaetes have also been studied to obtain a greater understanding of the effects of contaminants in the environment, such as heavy metals. In aquatic environments, heavy metals in particular are found bound to the sediment (Lee *et al*., 2000). Due to their close proximity with the sediment, and ingestion of such sediment, polychaetes may be useful as an indicator species to monitor levels of contaminants in the environment and in particular, the sediment (Mason *et al*., 1988; Moore and Dillon, 1992; Pocklington and Wells, 1992). Polychaetes are also well suited for this purpose due to their relatively small size, controllable diet and short life cycle (Hutchinson *et al.*, 1995). Polychaetes may therefore be used to predict the concentration of contaminants in the environment and provide information that could be used for conservation or environmental purposes (Mason *et al*., 1988).

The response of *N. acuminata* to the effects of heavy metal contaminants has been studied and is used as an indicator species of semi-polluted conditions (Reish, 1966). In this species, several contaminants have been used, to monitor effects on growth, reproduction and mortality (Pocklington and Wells, 1992) and accumulation of compounds can be observed in the body tissues (Pocklington and Wells, 1992). Heavy metals used in bioassay trials have included lead, mercury and zinc (Reish *et al*., 1976), copper (Pesch and Morgan, 1978), chromium (Oshida *et al*., 1981; Oshida and Word, 1982) and cadmium (Jenkins and Mason, 1988). The effects of radiation were also observed in *N. acuminata* (Harrison and Anderson, 1994a; 1994b). It has been observed that most pollutants tested caused some change in the reproduction of *N. acuminata*. Radiation was shown to limit reproductive success (Harrison and Anderson, 1994a; 1994b) and cadmium (concentration $10^{-8}$M $Cd^{2+}$) was found to completely eliminate reproduction following eleven weeks of exposure (Jenkins and Mason, 1988). Earlier studies on other heavy metals such as mercury and zinc, showed these to be toxic to the individual but adults were found to be more tolerant than juveniles (Reish *et al*., 1976).

Polychaete species are abundant in the environment, easy to sample, easy to maintain in the laboratory and have a life history length that is suitable for observing experimental effects (Pocklington and Wells, 1992). These traits hold true for *N. acuminata*; individuals easily adapt to laboratory conditions and do not require a period of acclimatisation before bioassays can be undertaken (Reish, 1985). Also, populations of this species only require a small amount of space to be housed and are easy to transport (Reish, 1980b). This makes *N. acuminata* an ideal species to use as a model organism to investigate the behavioural and evolutionary processes examined in this thesis.

### 1.4: Laboratory populations and culture conditions

Sexually mature male and female *N. acuminata* individuals can be separated and identified due to the presence of gametes in the coelomic cavity, which vary in colour between the two sexes (see Figure 1.3 below): sexually mature females are an intense yellow colour whereas males are white (Reish, 1957). A male and a female can also be distinguished from one another according to aggression as a male and a female will not fight each other if placed together in a crystallising dish (Reish, 1957). When used in short-term observational experiments using two individuals of the same sex, the two individuals can be distinguished from each other using the faecal matter pattern visible in the gut, which varies between individuals due to slight changes in feeding habits between individuals (Fletcher *et al*., 2009).

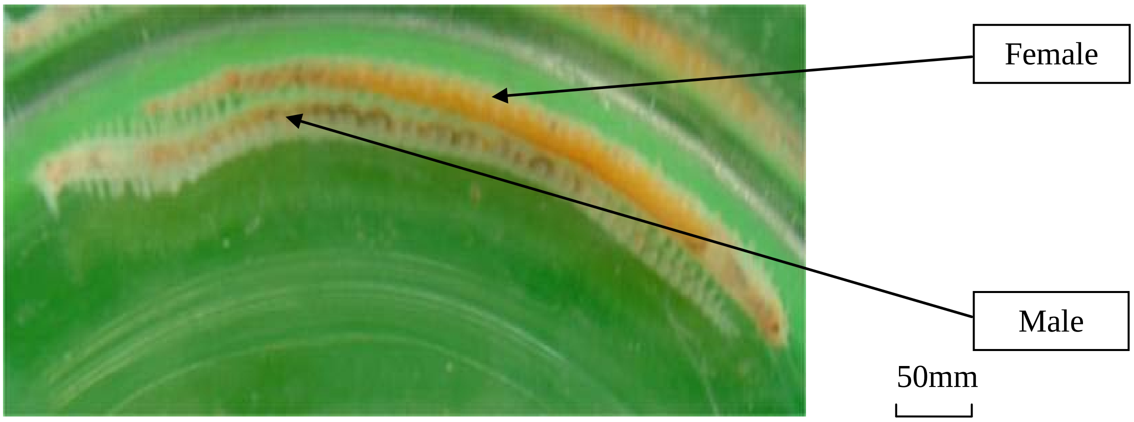


Figure 1.3: Colouration of male and female *N. acuminata* due to the presence of gametes in the coelomic cavity.

Populations maintained in the laboratory originate from four locations in the United States of America. The different populations of *N. acuminata* were collected from the coastal regions of North America by Dr. D.R. Reish. West Coast populations were collected from Los Angeles Harbour (‘Reish’ population), Newport Beach and the San Gabriel River in California. The East Coast population was collected from Alewife Cove, Connecticut. These populations are segregated in both the laboratory based at the

California State University, Long Beach (CSULB) and at the University of Hull and are henceforth referred to as Reish (R), Newport (N), San Gabriel (SG) and Connecticut (C). The number of stock tanks maintained in the laboratories at CSULB and at the University of Hull is detailed in Table 1.3 below. Separate populations are housed in aquaria tanks (180mm x 100mm x 140mm) containing approximately 100 adult individuals per tank, all at a salinity of 35-40‰. Although named the San Gabriel River, this is actually a concrete channel with a salinity of +30‰ (Hardege, personal communication) so this population is also maintained at a salinity of 35-40‰. The laboratory populations are fed three times a week on a diet of low protein non-processed compressed hay (rabbit food) obtained from Smith’s Animal and Pet Supplies (Castle Road, Hull) and *Enteromorpha spp.* algae. The laboratory room temperature is maintained at 18° C with a light:dark cycle of 16:8. The Reish population was established from six breeding individuals collected in 1964 (Reish, 1985) with the sex ratio of the specimens unknown (Reish, personal communication). Although Reish (1985) states that there does not appear to be any decrease in vitality of this populations, it could still be heavily inbred and this should be taken into account when analysing any data collected using this population. In addition to these four laboratory populations, one wild population was sampled from Los Angeles Harbour in 2008 (ratio of males to females unknown) and bred in the laboratory to produce a population with over two hundred individuals. It was originally thought that the Reish (R) population could no longer be sampled in the wild but individuals have been sampled very near to where the original Reish (R) population was sampled and will henceforth be referred to as LA Harbour (LA). Both R and LA populations were collected using a sediment bottle collector suspended from floating docks in the west basin of Los Angeles Harbour (Reish, 1957, Reish, personal communication). The harbour is divided into five regions: the east basin, west basin, Fish Harbour, Terminal Island and the Long Beach Naval Base (Reish *et al.*, 1980). When environmental studies commenced on the harbour area in 1951, there were high levels of organic content and the harbour was a heavily polluted body of water injected daily with untreated waste discharges (Reish *et al.*, 1980). In 1968, a pollution abatement programme was ordered by the Californian Regional Water Quality Control Board to ban the oil-refinery discharge (Reish *et al.*, 1980). In 1975, pollution abatement at the Long Beach Naval Base commenced and in 1978, fish cannery wastes from factories based at Fish Harbour were diverted to the Terminal Island Sewage Treatment Plant (Reish *et al.*, 1980). Also in 1978, the oil tanker S.S. Sansinena exploded in the outer harbour, causing a major ecological disaster. Biological, chemical and physical measurements taken subsequently indicated a crash in the benthic diversity, but the area is recovering following a clean-up operation and dredging of the area (Reish *et al*., 1980). Although there was a tanker explosion, due to the abatement programme, the harbour area is now classed as a less polluted body of water between the time of the R collection (1964) and the LA collection (2008) (Reish, 1986).

The date each population was collected, along with approximate numbers of specimens collected in each case can be seen in Table 1.3 below, including the number of stock tanks maintained at each facility. The sampling locations for each of the populations maintained in the laboratory are shown in Figure 1.4 on the next page. Experiments were carried out at the University of Hull unless otherwise stated and results in all chapters were analysed using SPSS version 16 (SPSS Inc., Chicago, Illinois, U.S.A.), apart from in Chapter 3 (section 3.3.1), where the statistical package R was used (R version 2.10.1).

Table 1.3: Collection details for the populations maintained in the laboratory with the number of specimens collected in each case, including the number of stock tanks of each population maintained at each facility.

| Population | Year sampled | Individuals collected | Stock tanks | |
|---|---|---|---|---|
| | | | CSULB | Hull |
| Reish (R) | 1964 | 6 | 7 | 4 |
| Newport (N) | 2005 | 400 | 4 | 3 |
| San Gabriel (SG) | 2003 | 300 | 3 | 3 |
| Connecticut (C) | 2002 | 300 | 3 | 2 |
| Los Angeles (LA) | 2008 | 300 | 3 | 2 |

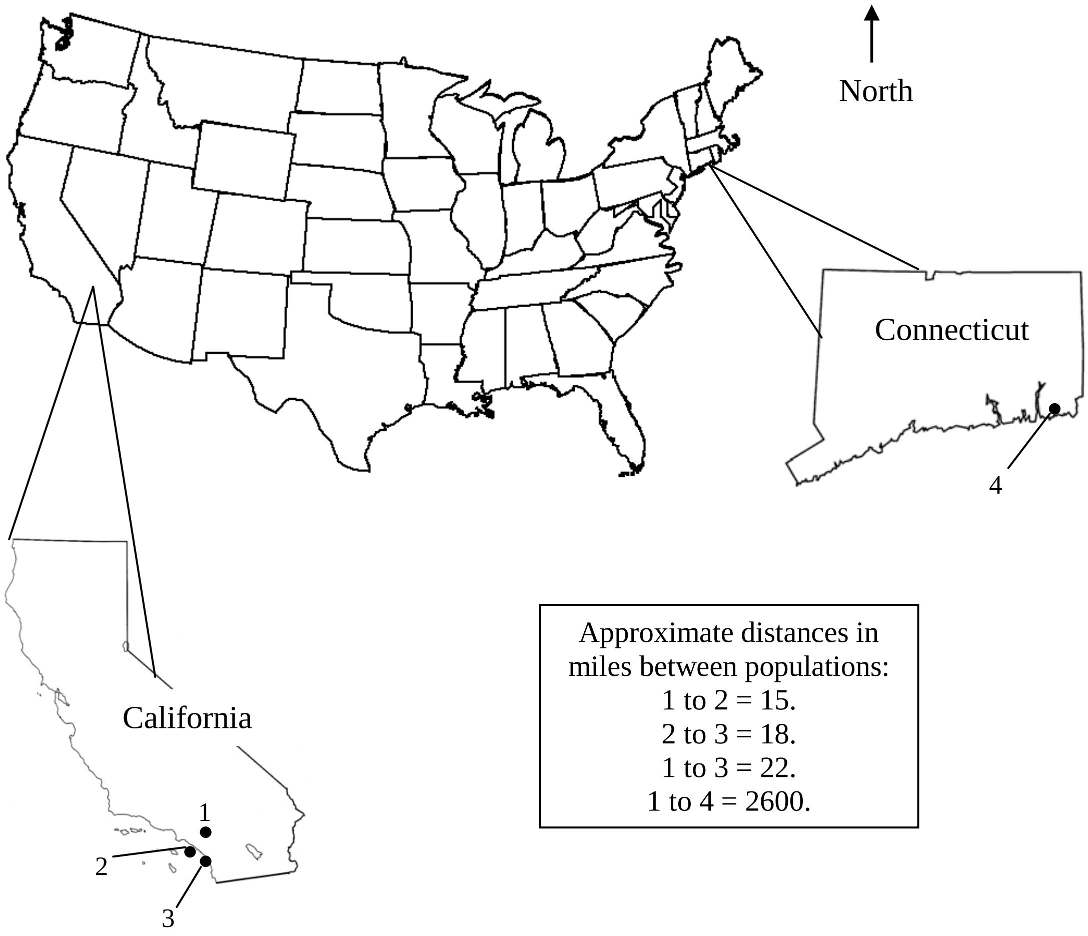


Figure 1.4: Population locations within North America. Sample locations for each population are denoted by: 1 = San Gabriel River (SG); 2 = LA Harbour (R); 3 = Newport Beach (N), all from California; 4 = Alewife Cove, Connecticut (C). The wild population Los Angeles (LA) was sampled at location 2, (the same as the R population).

Previous work has been undertaken on these laboratory populations to determine the molecular differences between the populations. Pesch and Pesch (1980) found that the R population had a diploid chromosome number of 18 and subsequently, this was also found to be the case in the N and SG populations (Weinberg *et al*., 1990). However, the diploid chromosome number of the C population was found to be 22 (Weinberg *et al*., 1990). Chromosome numbers were also documented in other Nereid polychaetes and are as follows: *Nereis limbata* diploid 20-30, *Platynereis megalops* haploid 14, *Nereis diversicolor* diploid 32 and *Perinereis cultrifera* diploid 34 (Pesch and Pesch, 1980). Differences in chromosome number can be used as indicators of reproductive incompatibility (Knowlton, 1993). Changes in chromosomes can play a role in evolutionary change, including the loss or gain of whole chromosomes but this may not influence any morphological change in a species (John, 1981). Such chromosome change may, but does not always, influence speciation and can act to isolate individuals reproductively with differences at the chromosome level (John, 1981).

The pairing behaviour both within and between populations of *N. acuminata* has also been examined, including both pre- and post-mating isolation trials to determine levels of reproductive isolation (Weinberg *et al*., 1990; Weinberg *et al*., 1992; Rodriquez-Trelles *et al*., 1996; Sutton *et al*., 2005). This is known as the final step before speciation and individuals can either be isolated by pre-zygotic barriers or by post-zygotic barriers (Snell, 1989). Pre-zygotic barriers include differences in behaviour, mating preferences (Snell and Hawkinson, 1983), mating season, fertilisation mechanisms (Snell, 1989) habitat and spawning synchrony (Palumbi, 1994). Post-zygotic barriers include genetic incompatibility, hybrid sterility and hybrids that are not viable (Snell, 1989). Pre-mating bioassays are generally simple and quick to undertake and are useful for sibling species where morphological differences may be minimal (Snell, 1989). In the experimental species *N. acuminata*, pre-mating isolation trials are suitable for study due to the initial pairing behaviour of a male and a female before the eggs are shed by the female. This behaviour is easy to observe and gives a good indication of compatibility. Weinberg *et al.* (1990) looked at the pre-mating isolation levels over the course of ten minutes using two populations from the Atlantic Ocean: Massachusetts (M) and Connecticut (C), and two from the Pacific Ocean: San Gabriel (SG) and Newport (N). Pairing formation was not observed between male and female individuals from the Atlantic and the Pacific, therefore showing evidence of significantly higher levels of reproductive isolation between populations compared to the pairing behaviour between male and female individuals from within each population. However, between the SG and the N populations, there were no observations of pre-mating isolation. The above ten minute trials were then repeated, again by Weinberg *et al.* (1992), this time using the San Gabriel (SG), Newport (N) and Reish (R) populations. High levels of pre-mating isolation were observed between R and both SG and N, however there were low levels of pre-mating isolation between SG and N. It was therefore concluded that speciation occurred in the R population in the laboratory following a founder event. Weinberg *et al*. (1992) also looked at the percentage of broods that produced healthy offspring between the R, SG and N populations (a post-mating isolation experiment). A normal percentage was found to be between 75-95%. When R individuals were paired with either SG or N individuals, the percentage of healthy offspring was equal to 0. However, when SG individuals were paired with N individuals, the percentage of healthy offspring was found to be 77%.

The above work by Weinberg *et al.*, (1992) has caused some controversy as it was suggested that speciation of the R population had occurred in the laboratory. The findings were therefore further discussed in the work by Rodriquez-Trelles *et al*. (1996). This work disagreed with Weinberg *et al*. (1992) and hypothesised that the two wild populations used in the study (SG and N) were not representative of the population from which the R population was originally sampled from. The study went on to investigate the genetic markers, genetic variability and divergence of the three populations. It was found that in 13/18 loci, the R population does not share any alleles with either SG or N and high genetic differences (D) were found to be as follows: SG to N = 0; R to SG = 1.75; R to N = 1.76. The genetic difference between the two sibling species *N. diversicolor*, a population from Germany, and *N. limnicola*, a species from the U.S.A., is 1.28. The study therefore concludes that these three populations formed separate species before the R population was sampled in 1964.

One final study (Sutton *et al.*, 2005) looked at the pre-mating isolation between populations of *N. acuminata*, using ten minute trials between three laboratory-based populations (SG, N and R) and three wild populations (Connecticut, Massachusetts and a population from Hawaii). Again, significant levels of pre-mating isolation were found between all population groups, except when individuals were paired with members of their own population. Further to this, D.H. Lunt and J.D. Hardege (unpublished) examined the nuclear and mitochondrial DNA from these populations. According to the nuclear DNA, the C population is likely to have diverged from the other three populations approximately ten million years ago. Examining the mitochondrial DNA, following the divergence of the C population, the SG population diverged from the R and N populations approximately seven million years ago. R and N have been found, using the mitochondrial DNA, to be very similar genetically, with little divergence. The Connecticut worms have also been observed to have black eyes, whereas individuals from the other populations, including the wild LA population, have red eyes (Reish, personal communication). There is still very little known about the new wild LA population, both in terms of behaviour and molecular composition.

If *N. acuminata* forms part of a species complex, this may impact the results observed previously in both behavioural and ecotoxicology tests. This has been found to be the case in ecotoxicology studies using the mussel *Mytilus edulis*, the most commonly used species for biomonitoring of water quality. This species is part of a species complex with *M. galloprovincialis* and *M. trossulus*; *M. trossulus* is commonly mistaken for *M.*

*edulis* (Lobel *et al*., 1990). In the study by Lobel *et al*. (1990), *M. edulis* and *M. trossulus* were collected from the same site in Canada and the body tissue analysed for twenty five different element concentrations (ppm dry weight), including Na, Zn, Cu, Ag, As, Ca, K, Al, Mg and Pb, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Due to the differences in metabolic rate between *M. edulis* and *M. trossulus*, the concentrations of the elements tested were found to vary, even though these two species were sampled from the same geographical area (Lobel *et al*., 1990). In the ecotoxicology tests described previously (Reish, 1966; Reish *et al*., 1976; Pesch and Morgan, 1978; Jenkins and Mason, 1988; Mason *et al*., 1988; Moore and Dillon, 1992), individuals were taken from the Reish (R) population housed in the laboratory of Dr. D.J. Reish at CSULB. However, another study looked at a population, sampled prior to experimentation, from the San Gabriel River, California (Oshida *et al*., 1981) and other works do not mention the original location of the individuals used (Oshida *et al*., 1982; Lee *et al*., 2000). Two further papers appear to have pooled results from the Reish population and another population from a supplier of test organisms (Harrison and Anderson, 1994a; 1994b). It is therefore clear that care needs to be taken over interpretation of ecotoxicology results in this species.

## 1.5: Aims

This thesis attempted to examine the role of aggressive relationships and male parental experience in mate choice in the polychaete *N. acuminata*.

Aggression was examined in *N. acuminata*: firstly, using females that have shed their eggs (spent) to determine if there was any aggression exhibited by such females when an egg mass is no longer present. It was expected that aggression would decrease as the female is no longer in the presence of her offspring and protection of her investment was no longer required. Secondly, the effect of male aggression on pairing behaviour was examined to determine if such aggression should be taken into account in studies looking at mate choice and aggression in females. It was expected that males aggression would cause pair time with females to increase following a fight as female *N. acuminata* have been shown to prefer good fathers over dominant males (Fletcher *et al*., 2009). Thirdly, the aggression between males was examined over a period of time, with repeat fights observed, to determine if there was any memory for aggressive encounters and any evidence for the existence of a dominance hierarchy in this species. It was expected, as nereid polychaetes are capable of learning (Evans, 1966b; Dyal, 1973), and recognition of a previous opponent would decrease the costs and risk associated with aggressive behaviour (Caldwell, 1985), that less aggression would be observed in subsequent fights between individuals.

The levels of intrasexual aggression between the four laboratory populations were also examined, both in males and females, to determine if there were any similarities or differences in aggression (within or between populations), supporting the evidence of divergence between these populations. Genetic divergence has been observed between the four populations Reish (R), Newport (N), San Gabriel (SG) and Connecticut (C) examining differences in chromosome number (Pesch and Pesch, 1980; Weinberg *et al*., 1990), comparison of shared alleles (Rodriquez-Trelles *et al*., 1996) and both mitochondrial and nuclear DNA (D.H. Lunt and J.D. Hardege, unpublished). Divergence in populations can be expressed in changes in behaviour (Dunbrack and Clarke, 2003) and communication signals which deviate from the population norm have the potential to be ambiguous and lead to an escalation of aggression (Maynard Smith and Riechert, 1984; Dunbrack and Clarke), 2003. Behavioural divergence has not been examined before in *N. acuminata* in terms of the expression of intrasexual aggression. It is therefore expected, as divergence can lead to changes in behaviour and escalation of fights can occur due to such divergence, that aggression between populations would be higher than aggression exhibited within populations.  The new wild Los Angeles Harbour (LA) population (sampled in 2008) was also compared to the existing laboratory maintained Reish (R) population to determine if there were any differences in aggression between these two populations and whether these results suggest that these two populations should be treated as separate, providing an indication of divergence. The LA population is a new addition to the populations maintained in the laboratory but was sampled from the same location as the R population.  It was expected that aggression between these two populations would be higher due to the variation in environmental conditions experienced by these two populations, leading to population divergence.  It is possible that distinct populations of *N. acuminata* should be classed as sibling species, forming a species complex.  As *N. acuminata* is used in ecotoxicology testing and water quality monitoring, the existence of a species complex in *N. acuminata* must be taken into account when performing such bioassays as different populations may respond differently to testing, due to potential differences in metabolic rates, growth rates and heavy metal tolerances for example (Lobel *et al*., 1990).

The levels of male aggression were also compared to the levels of female aggression to determine if one sex displays more aggressive behaviour, helping to further understand the role of sexual selection in this species.  Due to female death following reproduction and the ability of males to reproduce again, female mate choice is likely to occur in *N. acuminata* due to the higher reproductive investment made by females (Starczak, 1984). As female mate choice and male aggression has been documented in *N. acuminata* (Starczak, 1984; Fletcher *et al*., 2009), it was therefore expected that male aggression would be higher than female aggression.

Following on from the observations of aggressive behaviour between individuals from different populations, the interactions between males and females from the four laboratory populations and the new wild population were examined to determine if pre-mating isolation occurred, providing further evidence of divergence in this species.  Pre-mating isolation is considered to be the final step before speciation (Snell, 1989).  It was expected that the number of individuals forming a pair bond between the four laboratory populations would be lower between populations than within them due to divergence of the communication signals within each population caused by variations in habitat conditions at the location that each population was originally collected from.  If pre-mating isolation is evident between individuals from the R and LA populations, this would provide further evidence for the theory that the R population speciated in the laboratory (Weinberg *et al*., 1990). It was therefore expected that few pairs would be formed between these two populations due to the variations in habitat conditions experienced by each population.

The incidence of filial cannibalism has been previously mentioned to occur in *N. acuminata* (Oshida *et al*., 1981) but such behaviour has not been fully examined. The effect of the presence of a cannibalistic male on aggressive interactions between males was examined, comparing cannibalistic males to both inexperienced males (that have not reproduced) and experienced males (ones that have reproduced). It was expected that as males consuming eggs should be in good physical condition, a reduction in the occurrence of escalated aggression would be observed. The presence of a male potentially in better condition would alter the Resource Holding Potential (RHP) of that male, causing there to be asymmetry between the opponents. Such asymmetries due to differences in the RHP of opponents can lead to the quick resolution of fights (Maynard Smith, 1974). Female interactions with cannibalistic males were also observed. As sole male parental care occurs in this species (Reish, 1957) and females make a larger gametic investment than males (Starczak, 1984), the female should select a male in good physical condition, capable of rearing her brood (Hoelzer, 1989). Female mate choice trials were therefore observed to determine if a female could detect that a male has cannibalised an egg mass under his care and if so, whether the female would still form a monogamous pair bond with such a male. As females should select a male based on his ability to care for her brood to avoid her reproductive investment being wasted (Hoelzer, 1989), and as males that consume eggs may be in better physical condition (Klug and St. Mary, 2005) and less likely to cannibalise eggs during future egg care (Manica, 2010), it was expected that females would not avoid pairing with a male that had previously cannibalised an egg mass.

As yet, the process of sexual selection in *N. acuminata* is not fully understood. Although female mate choice has been exhibited, it is still not clear how males confer to females the reproductive advantage of previously caring for an egg brood. Previous trials have shown that females will alter their initial choice based on the presence of a previous loser masked with water from males caring for eggs (Storey, 2006; Fletcher *et al*., 2009). It was an aim of this thesis to further observe the chemical nature of experience by undertaking conditioned water trials from a variety of sources and observing their effects on mate choice trails where a winner and loser of female choice had already been established. The conditioned water used in masking trials was obtained from females, juveniles, inexperienced males, males caring for eggs and both new and old experienced males. As females have been shown to prefer experienced males over inexperienced males, it was expected that males placed in conditioned water from new and old experienced males would alter subsequent female choice, as would the conditioned water from males caring for eggs. It was expected that the conditioned water from females, juveniles and inexperienced males would not alter subsequent female choice as these should not alter the initial choice of mate made by the female. Aggression between individuals following the use of conditioned water was also observed to determine if there was any change in aggression following the masking of a male with conditioned water from various sources. Individuals use assessment strategies to determine the RHP of their opponent and the likelihood of winning a fight (Maynard Smith, 1974). Placing males in conditioned water from females, juveniles and inexperienced males was not expected to alter the aggressive behaviour observed between individuals, as the RHP of the masked opponent (an inexperienced male) should not be altered by the conditioned water. However, conditioned water from males caring for eggs and both new and old experienced males was expected to alter the aggression observed between two opponents as the RHP of the masked male would have changed due to the ‘presence’ of a male undertaking egg care or an experienced male.

The final aim of this thesis was to attempt to demonstrate ‘adoption’ in this species. Previously unpublished work has indicated that males care for eggs fertilised by another male (Sutton, 1998) but has not been performed under experimental conditions and has not taken into account the reproductive state of the male, comparing those that have undertaken parental care previously (experienced) to those that have not (inexperienced). This was therefore examined here, comparing such males to see if there was evidence that males care for eggs fertilised by another male. Females have been shown to select mates based on parental care abilities in *N. acuminata* (Fletcher *et al*., 2009). Adoption in inexperienced males may therefore be a way for individuals to gain parental experience. It was expected that care of eggs fertilised by another male would occur in inexperienced males, but that experienced males would not undertake such care as they do not gain benefits from such behaviour as they have already successfully reared offspring.

As previously mentioned, the more we know about the evolutionary and behavioural processes in the polychaete *N. acuminata*, and the interactions between the populations, the more we can apply that knowledge to undertake environmental studies, using this species as an indicator of pollution and water quality monitoring. We can also use this species as a model for evolution as it has an easily adaptable life history, a relatively short life cycle and is convenient to use for behavioural bioassays. Observations are easy to make and experiments relatively simple to conduct at each stage of the life cycle.

# Chapter Two:

# Aggression and pairing behaviour in *N. acuminata*

## 2.1: Experimental reasoning and aims

Aggressive behaviour, also described as agonistic behaviour, is defined as any behaviour associated with a contest between individuals, which often includes initiation, attack and eventual retreat or death (King, 1973). Inter- and intraspecific aggression has been observed in both terrestrial and aquatic species, such as birds (Sandell, 1998), insects (Polizzi and Forschler, 1999), crustaceans (Hazlett, 1968), fish (Sale, 1972) and marine invertebrates (Reish and Alosi, 1968). Such aggression may be due to competition for resources e.g. for food (Commito and Shrader, 1985), space (Chadwick, 1987) or mating opportunities (Parker, 1974); for defence of territory (Reish and Alosi, 1968), offspring (Bridges *et al.*, 1996) or nest sites (Itzkowitz, 1990). Individuals may also engage in aggressive interactions for social rank, dominance, to prevent other individuals from mating or to be the first individual to mate with a particular partner (Karvonen *et al.*, 2000).

In the lace bug *Gargapha solani*, females display aggression in order to protect their offspring (Tallamy, 1982). In the willow ptarmingan *Lagopus lagopus* females are aggressively territorial against intruding females (Hannon, 1984). Several crustacean species have been also shown to be aggressive in order to compete for resources. Both male and female individuals of the shore crab *Carcinus maenas* display aggressive behaviour (Sneddon *et al.*, 1997) but female aggression is less intense (Huntingford *et al.*, 1995). Both male and female individuals of the American lobster *Homarus americanus* will fight territory intruders of either sex, but males have been shown to be more competitive (Peeke *et al.*, 1998). Male individuals not only require shelter for protection from predators, like females, but also for reproduction (Peeke *et al.*, 1998). *H. americanus* have also been shown to establish and maintain dominance hierarchies (Karavanich and Atema, 1998). Individuals of the shrimp species *Loligo pealii* form dominance hierarchies where males will guard females and use arm waving to threaten approaching males and this often escalates to rushes, tail bumping and dark spot display (a further warning sign) if the approaching male persists (Arnold, 1962). In polychaetes, aggression involves eversion of the proboscis, thrust forward toward the opponent (Reish, 1957), with the jaws used for biting or grasping (Weinberg *et al.*, 1990). Polychaetes known to engage in aggressive encounters include *Phragmatopoma lapidosa californica* (Pawlik *et al.*, 1991), *Platynereis bicanaliculata* (Woodin, 1974), *Nereis virens* (Commito and Shrader, 1985), *Nereis diversicolor* (Davey, 1994), *Platynereis dumerilii* (Evans, 1973), *Nereis pelagica* (Clark, 1959), *Nereis grubei*

(Reish and Alosi, 1968), *Nereis fucata* (Evans, 1973), *Nereis latescens* (Reish and Alosi, 1968) and the experimental species *N. acuminata* (Reish, 1957).

This chapter is divided into three separate studies on the aggressive behaviour of *N. acuminata*: aggression between sexual mature females and females that have laid their eggs (spent females), the effects of aggression on pair formation, and the effect of previous bouts between males on subsequent aggressive interactions.

### 2.1.1: Aggression in sexually mature and spent females

Females can compete for access to reproductive resources such as nest sites, food, high quality mates and in defence of offspring (Berglund *et al*., 1992). Females are expected to compete for mates if high quality males are in short supply (Ridley, 1978) and a female should be more aggressive towards another female intruding into her mate's territory/showing interest towards her mate, according to the investment guarding hypothesis (Yasukawa and Searcy, 1982). When one sex makes substantial contributions of parental investment, sexual selection can favour the evolution of adaptations that protect the investment (Trivers, 1972 cited Yasakawa and Searcy, 1972). Aggression in females can be heightened during the breeding season (Slagsvold, 1993) and intruders to the nest area may affect the resident female in a number of ways. Females that settle nearby with a mate can increase the competition for resources, but intruding females may also destroy offspring, parasitize the nest, displace the resident female or her mate and/or settle with the resident female's male, therefore decreasing the paternal care extended to her offspring (Slagsvold, 1993). Female willow ptarmigan *Lagopus lagopus* monogamously pair with males and have been found to chase and attack other female intruders to the territory, preventing unmated females from settling (Hannon, 1984). In the red-winged blackbird *Agelaius phoeniceus* females display more aggression towards females soliciting their mates (Yasukawa and Searcy, 1982). In the great tit *Parus major*, resident females are aggressive towards intruding females but not towards intruding males (Slagsvold, 1993). Such aggression from females has been suggested to be important in maintaining the monogamous relationship between males and females (Wittenberger and Tilson, 1980) and also secures full male assistance with caring for offspring (Davies, 1989 cited Sandell, 1998).

The aggressive behaviour of individuals has also been shown to change when offspring are present and over the course of the breeding season. In the scissortail sergeant *Abudefduf sexasciatus*, males care for eggs solely and are only aggressive during this period (Manica, 2010). Several studies have shown that whilst in the presence of offspring, females also increase their aggression levels in order to protect the reproductive investment, known as maternal aggression (Tallamy and Denno, 1981). Such aggression has been observed in the white tailed ptarmigan *Lagopus laucurus*, with females observed to exhibit heightened aggression towards other conspecific females whilst caring for eggs (Martin *et al*., 1990). Females of the stomatopod *Gonodactylus bredleni* solely care for egg masses and defend nest cavities against predators and conspecifics only when caring for such masses (Montgomery and Caldwell, 1984). Individuals caring for offspring also increase the intensity of aggression as the offspring get older and the probability of survival increases (Jaroensutasinee and Jaroensutasinee, 2003).

In *N. acuminata*, a spent female has been observed to fight a sexually mature female if one is introduced to the dish that houses the male caring for her eggs (Reish, 1957), presumably to prevent the female from attempting to mate with the male guarding her eggs, therefore protecting her reproductive investment. As females of this species die following reproduction, the survival of eggs depends entirely on the male and females should protect their reproductive investment, not only by selecting a good quality male to care for her eggs, but also by preventing the male from reproducing with another female and deserting or cannibalising the spent female's eggs. The first aim of this chapter was to determine if spent females engage in aggression without the presence of a male caring for their eggs and to determine if spent females show any aggressive behaviour once they are separated from their own egg mass. As aggression levels in other species have been shown to only increase when protecting eggs, it was expected that the aggression levels shown by spent females would be less than that between sexually mature females as the female is no longer in the presence of the egg mass.

### 2.1.2: The effect of male aggression on pair formation

One of the broadest areas of research concerning female mate choice has been regarding dominance hierarchies in species with male to male contests. Females select for dominance in many species (Searcy, 1982) and this has been observed in mammals, fish, birds, insects and other invertebrates (Forsgren, 1997). This has been hypothesised to occur for a number of reasons. It may be that dominant males possess a greater ability to provide breeding resources such as territory and female protection (Moore *et al*., 2003) and dominance may also be a good indication of reproductive success in males (Searcy, 1982). Dominant males are likely to be larger in size and this may indicate that they are superior at fighting for access to mates (Bisazza *et al.*, 1989) or that they are better able to provide protection against predators (Takahashi and Kohda, 2001). Females have also been found to "eavesdrop" on aggressive contests between males to gain information regarding mate quality, as observed in the female Siamese fighting fish *Betta splendens* (Doutrelant and McGregor, 2000). This ensures that females mate with high-quality males (Forsgren, 1997) as success in contests is indicative of good male condition (López *et al.*, 2002). In the shore crab *Carcinus maenas*, females mate with dominant males more often whereas subordinate males have to use alternative breeding strategies, such as sneaking matings with females (Van der Meeren, 1994).

However, in some species, although male to male contests and the formation of dominance hierarchies can be observed, females do not always select for the dominant individual (Moore *et al.*, 2003). In the rock shrimp *Rhynchocinetes typus*, although a male dominance hierarchy exists, it has been found that females will mate firstly with subordinate males to prompt further displays by dominant males (Thiel and Correa, 2004). It is hypothesised that this further display acts as a reassurance for females, ensuring that females select to mate with winners and therefore good quality males (Thiel and Correa, 2004). Also, in the three-spined stickleback *Gasterosteus aculeatus*, females show no preference for dominant males over subordinate males, selecting males that were less aggressive (Ward and FitzGerald, 1987). However, dominance may not be the trait selected for by female mate choice. In species with male parental care, the ability to raise offspring may be the deciding factor in mate choice, with good fathers preferred (Wong, 2004). Hatching success must be heavily reliant on male care for females to select mates based on their parental care ability and therefore, the selected trait conferring the signal to the female must be reliable (Hoelzer, 1989). Dominance may not provide such a reliable indication of parental care ability (López *et al.*, 2002) and dominant males do not always make better fathers (Wong, 2004). Hatching success in the sand goby *Pomatoschistus minutus* has been shown to be dependent on sole paternal care and males with nests containing eggs are preferred over those with empty nests (Forsgren *et al.*, 1996). Added to this, dominant males were not found to be better at egg care and females gained no direct benefits from mating with such males (Forsgren, 1997). Finally, female fifteen-spined sticklebacks *Spinachia spinachia*, exhibit mate choice for males with higher fanning abilities, necessary for the oxygenation of eggs during parental care (Östlund and Ahnesjö, 1998).

Previous studies have examined the dominance relationships in *N. acuminata* (for example Starczak, 1984; Fletcher *et al*., 2009) but these studies do not appear to have taken into account whether the aggressive bouts themselves have any subsequent effect on pairing behaviour when males are placed with females. The outcome of aggressive interactions may influence the ability of each male to attract a mate and may affect female choice (Kangas and Lindström, 2001). This could therefore have an impact on results obtained in studies looking at the aggressive interactions between males, for example if males are returned to fight with one another. The second aim of this chapter was to investigate the effects of an aggressive bout on the subsequent time it takes for males to form a pair bond with a female, seen when males and females lie alongside each other 'head to tail' and start to construct a new mucous tube. It was expected that, as male parental care is vital in this species due to female death following reproduction (Reish, 1957), that pairing with males capable of performing such care would be more important to females than male fighting ability. Females have previously been shown to select for males experienced in parental care, rather than males showing overt aggression (dominant males) (Fletcher *et al*., 2009). It was therefore expected that the occurrence of an aggressive interaction would change the subsequent pairing time between a male and a female, increasing the time taken for a male and a female to form a pair bond.

The third aim of this chapter was to determine if there is a reduction in the time taken for a male and a female to form a pair bond if they are familiar with each other; if they have previously formed a pair bond. Mate recognition has been observed in other invertebrate species. In the banded shrimp *Stenopus hispidus*, individuals were found to behave differently when interacting with previous mates compared to interactions with strangers; previous mates display less courtship behaviour towards each other (Johnson, 1977). In the stomatopod *Gonodactylus bredleni*, a male and a female will share a nest site during reproduction (Caldwell, 1992). Once reproduction is complete, males will search for a new dwelling and will compete for this resource, evicting the resident (male or female) (Caldwell, 1992). If a male does not recognise the female that he has mated with, he may jeopardise his offspring. Under experimental conditions, male stomatopods never attacked a female with which they had copulated with but did attempt to evict unfamiliar nesting females (Caldwell, 1992). As mate recognition can lead to reduced courtship displayed between individuals (Johnson, 1977), it was expected that the time for familiar individuals to form pairs would be lowered in subsequent interactions in *N. acuminata*.

### 2.1.3: The effect of previous aggression between males on subsequent interactions

The formation of dominance hierarchies can be maintained when individuals that have previously fought are able to recognise each other (Gherardi and Teidemann, 2004). Recognition occurs when each individual in a group can be discriminated from every other individual based on a unique set of cues that define that individual (Karavanich and Atema, 1998). Individual recognition has been demonstrated in the hermit crab *Pagurus longicarpus*; individuals were more aggressive during fights when placed with unfamiliar opponents (Gherardi and Teidemann, 2004). Recognition of previous opponents has also been shown in male *Homarus americanus* lobsters, where previous encounters are remembered and the subsequent aggressive response is lowered (Karavanich and Atema, 1998).

The outcome of prior aggressive interactions can influence an individual's behaviour in subsequent interactions. Winning or losing fights can increase or decrease various measures of fighting motivation and affect the ability of the individual to win in a subsequent encounter. Winners of fights may keep winning, and losers keep losing (Collias, 1943 cited Jackson, 1991). In the pumpkinseed sunfish *Lepomis gibbosus*, prior winners of a fight defeated unfamiliar prior losers (Beacham and Newman, 1987). Males of the Norway lobster *Nephrops norvegicus* decrease their fight duration when encountering a familiar male a second time and the aggression displayed is strongly reduced (Katoh *et al*., 2008). Prior aggressive interactions have also been found to have a lasting affect (at least 48 hours) in the cyprinid fish *Rivulus marmoratus*, where winners win in subsequent fights and losers go on to lose again (Hsu and Wyatt, 1999). As escalated or prolonged fight pose a considerable risk to both opponents, recognition of former opponents may also reduce this risk of injury and reduce the costs associated with aggression (Caldwell, 1985).

Several studies have also been conducted into the learning ability of polychaetes and nereid polychaetes are thought to have the highest level of complexity among the different polychaete genera (Dyal, 1973). Nereids are known to use the rapid withdrawal reflex as a defence mechanism in response to sudden light changes or in the presence of a predator (Evans, 1963). It has been found that nereids can habituate with repeated exposure to stimuli and no longer use the withdrawal reflex; this is regarded as the simplest form of learning (Evans, 1966b). Habituation in *Nereis spp.* is said to be both a slow (Evans, 1966c) and short-lived process (Evans, 1966b). Examples of such habituation have been observed in *Nereis diversicolor*, *Nereis pelagica* and *Nereis virens*.

In *N. diversicolor*, individuals were observed to avoid a punishment at the end of a perspex tube (Evans, 1966c). In *N. pelagica* individuals fail to respond to light trials with the rapid withdrawal reflex after a number of trials (Clark, 1960). In *N. virens*, individuals also learn to avoid punishment but only if reinforced with a food reward at the end of an arm in a T-maze. If no reward was offered, there was no preference for either arm (Evans, 1966a). If nereids are able to learn, individuals may be able to use information acquired in prior aggressive interactions and use this information in subsequent fights with the same individual. Although the ability of nereid polychaetes to learn has been demonstrated (Evans, 1966b; Dyal, 1973), in *N. acuminata*, the ability of individuals to learn in terms of recognition of specific individuals and aggressive behaviour is unknown.

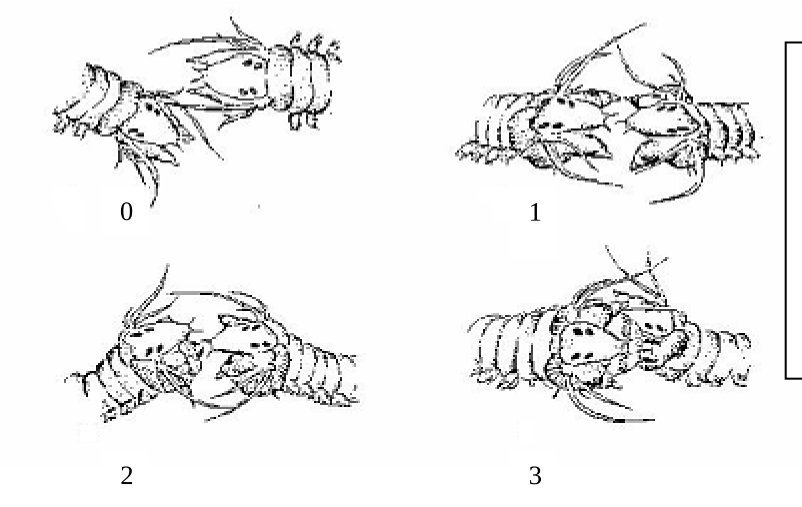
The final aim of this chapter was to determine if previous aggressive encounters between individuals affects their behaviour when they interact subsequently, observing if there were any changes in the levels of aggression recorded. This may have an impact on results obtained in studies looking at the aggressive interaction between males, for example if males are returned to fight with one another. The aggression displayed may not be due to the variable being tested but instead due to the recognition of a previous opponent, affecting the results. Any effect of previous aggressive encounters on subsequent encounters between individuals could also indicate that a dominance hierarchy is formed in *N. acuminata*. As nereid polychaetes are capable of learning (Evans, 1966b; Dyal, 1973) and recognition of former opponents would reduce the risk of injury and lower the costs of displaying aggression towards an opponent (Caldwell, 1985), it was therefore expected that aggression would decrease in subsequent interactions between *N. acuminata* males.

## 2.2: Methodology

All individuals used in this chapter originated from the Newport population. Experiments in this chapter were conducted at the laboratory at CSULB.

### 2.2.1: Aggression in sexually mature and spent females

Two females were placed in a crystallising dish (50mm diameter) containing 40ml of clean sea water (25mm depth of water, 49.09 $cm^3$ volume of water). Sexually mature individuals measure approximately 50mm (Pettibone, 1963) and individuals were placed in this amount of water for a short period of time only, making the amount of water used suitable for the study. Using the adapted Reish and Alosi (1968) aggression score detailed in Figure 2.1 below, an aggression score was taken over a fifteen minute period. Aggression was only recorded when both individuals had head to head contact and the experiment was initiated following frontal contact between the two individuals. Therefore, the aggression score is not a score of individual aggression but of the interaction between the two individuals. In previous studies, interactions between individuals of the same sex were observed for aggression from two minutes (Reish and Alosi, 1968) to a maximum of five minutes (Fletcher *et al.*, 2009). A fifteen minute observation period was used in this thesis to provide a good indication of the aggression, if any, displayed between individuals.



Score:
0 = No fighting response.
1 = Avoid contact and palpi may be flared.
2 = Fighting position assumed, palpi flared and jaws visible (eversed).
3 = Severe aggression with obvious attacks, biting and grasping.

Figure 2.1: Behaviours used to score the level of aggression between individuals, adapted from Reish and Alosi (1968).

The highest aggression level reached for each replicate was noted in each case (level 0, 1, 2 or 3) to control for any effects of sustained aggressive contact between the two individuals. Females in two different reproductive states were compared: spent females were placed with spent females (n = 10 fights), spent females were placed with sexually mature females (n = 10 fights) and finally an aggression score was also taken between two sexually mature females that had not yet shed eggs (n = 10 fights). Spent females were used for one replicate only on the day her eggs were observed in the parental tube as spent females only live for approximately a day after eggs are shed. Spent females are green in colour compared to the yellow colouration of sexually mature females. The total number of replicates equalled 30.

### 2.2.2: The effect of male aggression on pair formation

Two sexually mature, size-matched males were initially selected at random from the population tank and differentiated by the observer as male 1 and male 2 by the difference in individual gut contents visible in the digestive tract (Fletcher *et al.*, 2009). Each male was placed in a separate crystallising dish (50mm diameter) containing 40ml of sea water with a sexually mature female (also selected at random). The time taken for pair formation to occur with each male was noted. This was seen when a male and a female lie alongside each other ‘head to tail’. The two males were then immediately placed in another crystallising dish (50mm diameter), again with 40ml of sea water and an aggression score was taken of the interaction between the two males over fifteen minutes using the scoring system detailed in section 2.2.1, again, with the aggression scored as an interaction between the two individuals. In each case, the highest level of aggression reached (level 0, 1, 2 or 3) was noted. Following this, each male was returned to the crystallising dish containing the female and the time to form a pair bond was again noted (n = 25 fights). Each part of the trial was performed in succession on the same day when individuals from the population were found to be sexually mature.

The above protocol was then repeated, again using randomly selected males but after the fifteen minute aggression score was taken, males were separated and placed with two new females that they had not encountered before (n = 25 fights). This was undertaken to observe if the time taken to pair form was affected by encountering a previously unknown female. In all experiments, individuals were only used in one trial and not used again. Individuals were only used in trial when they had reached sexual maturity in population tanks. The total number of replicates equalled 50.

### 2.2.3: The effect of previous aggression between males on subsequent interactions

Two sexually mature, size-matched males were selected at random and placed in a crystallising dish (50mm diameter) containing 40ml sea water. The two males were noted as male 1 and male 2, differentiated using the individual gut contents (Fletcher *et al.*, 2009). A fifteen minute aggression score was then taken with focal observations made every 30 seconds, (section 2.2.1). In each case, the highest level of aggression reached was noted (level 0, 1, 2 or 3). The two males were then separated and placed in crystallising dishes (50mm diameter) containing 40ml of fresh sea water. The males were then returned to fight two hours after the first fight in the dish and the fifteen minute aggression score was repeated and any dominant male noted. Following the fight, males were again separated and placed in fresh sea water with a small pellet of low protein rabbit food. The aggression score was repeated once more after a period of 24 hours from the initial placing in the crystallising dish and again, the dominant male was noted. The total number of interactions equalled 60 (2 individuals observed on 3 separate occasions with n = 20).

2.3: Results

2.3.1: Aggression in sexually mature and spent females

Figure 2.2 below shows a summary of the highest level of aggression observed in each case when comparing females from the different reproductive states: spent versus spent (SS), spent versus mature (SM) and mature versus mature (MM).

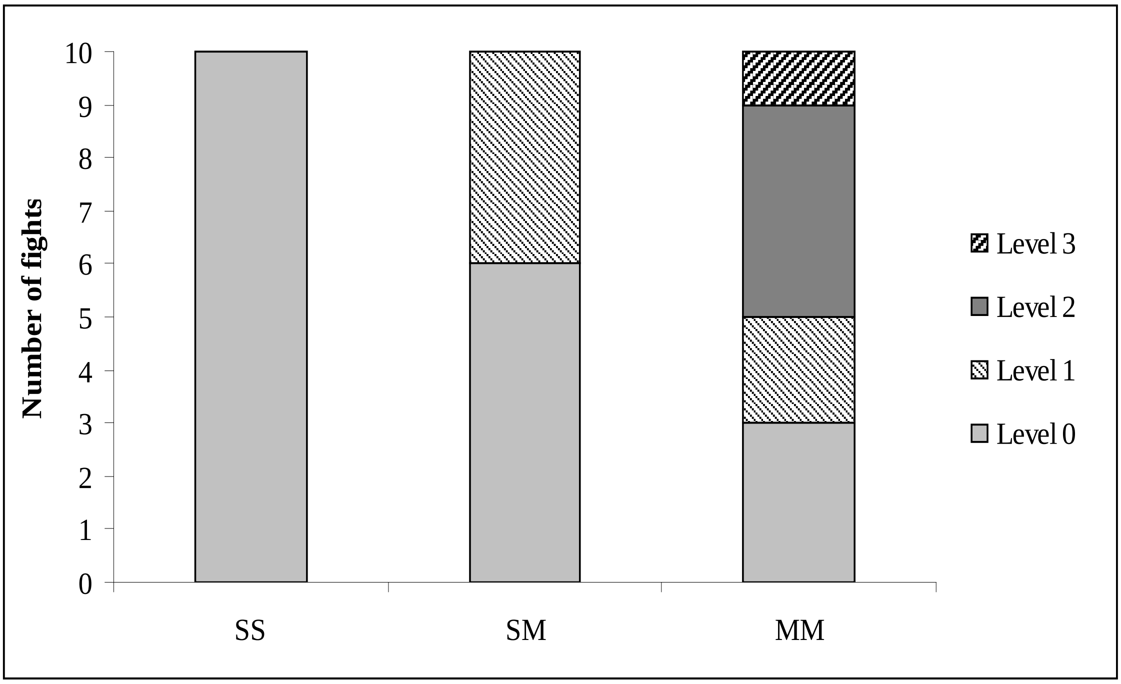


Figure 2.2: The number of fights reaching each aggression level comparing the three combinations of females; spent versus spent (SS), spent versus mature (SM) and mature versus mature (MM).

The results were analysed using the non-parametric Kruskal-Wallis test as aggression scores were compared as ranked data. There were significant differences in the aggression scores between the three combinations of females ($\chi^2/_2$ = 11.587, d.f. = 2, P = 0.003). As detailed in Zar (1996), post-hoc non-parametric multiple comparisons following the Kruskal-Wallis test were used to compare each combination of females: SS, SM and MM. Using a standard error of 26.926 and the critical value $q_{0.05,\infty,3}$ = 3.314, there were no significant differences between SS and SM (q = 2.321) or SM and MM (q = 2.043). However, a significant difference in aggression was found when comparing SS and MM (q = 4.364).

2.3.2: The effect of male aggression on pair formation

As shown in Figure 2.3 below, mean pre- and post-fight pair times for males placed with familiar females and unfamiliar females were recorded. There was a decrease in the mean pairing time when males were placed with familiar females following an interaction with another male.

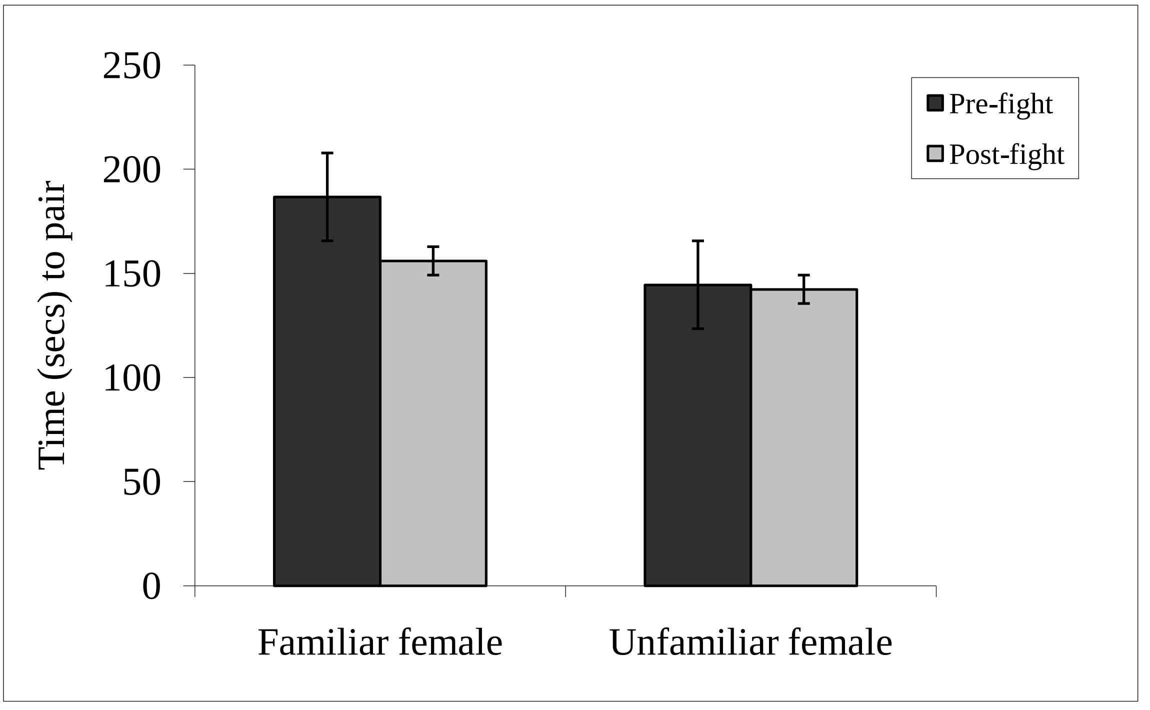


Figure 2.3: The mean time and standard error for a male and a female to form a pair bond prior and subsequent to an aggressive interaction with another male, placing the male either with a familiar or an unfamiliar female. Each bar represents 50 interactions (male 1 and male 2 pooled data).

The pairing times for male 1 and male 2 were pooled for each observation, giving a sample size of n = 50 for each pairing interaction (pre-fight with familiar female, pre-fight with unfamiliar female, post-fight with familiar female and post-fight with unfamiliar female). Pre- and post-fight mean pairing times for males placed with familiar and unfamiliar males were then analysed using a repeated measures ANOVA. Mauchly's test for sphericity was not significant (W = 1.000, P = 1.000), showing that the results do not violate the assumption of sphericity and no adjustment of the results of the repeated measures ANOVA is necessary. There were no significant differences found between the pairing times of males after an aggressive bout (F = 0.715, P = 0.400), whether the male was placed with a familiar female or an unfamiliar (new) female (F = 2.073, P = 0.153).

2.3.3: The effect of previous aggression between males on subsequent interactions

As shown in Figure 2.4 below, the results show that after the initial fight and period of separation, the occurrence of fights reaching level 2 and level 3 decrease for both subsequent fights and the occurrence of fights reaching level 1 increases, as more fights only reach this level and do not intensify.

To compare the highest levels of aggression found for each of the three different fights, the results were analysed using the non-parametric Friedman test which takes into account repeated measures. There were no significant differences found between the levels of aggression recorded ($\chi^2/_2$ = 4.769, d.f. = 2, P = 0.092). Although there is a decrease in level 2 and level 3 aggression for fights 2 and 3, these changes are not significant.

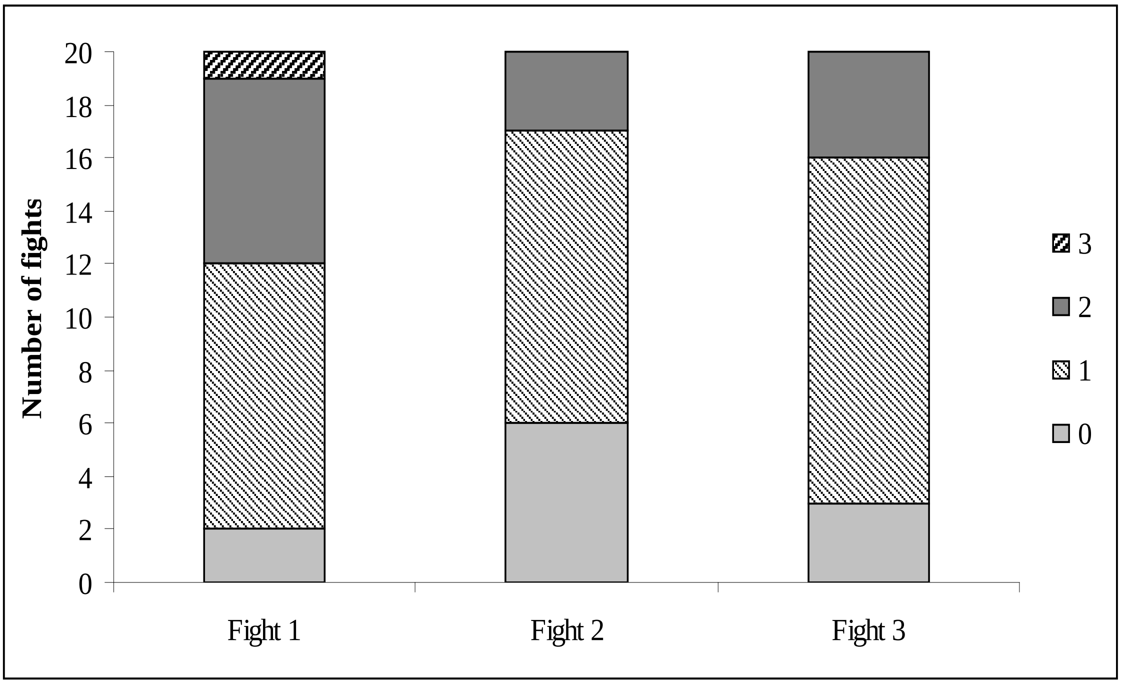


Figure 2.4: The total number of fights reaching each level of aggression for the initial fight (fight 1), the second fight after 2 hours (fight 2) and the final fight after 24 hours (fight 3). The number of interactions for each fight = 25.

## 2.4: Discussion

### 2.4.1: Aggression in sexually mature and spent females

Aggression has been observed to change in females during the course of the breeding season (Slagsvold, 1993) and whilst females are in the presence of their offspring (Manica, 2010). It is also possible that changes in parental behaviour are exhibited as the reproductive value of the parent or the offspring changes (Tallamy, 1982). In the lace bug *Gargapha solani*, females are more aggressive as their offspring reach maturity (Tallamy, 1982). Females should protect their reproductive investment from other females to prevent the male from mating with an intruding female to protect the parental care investment that males provide (Slagsvold, 1993). Although females die following reproduction, placing their lifetime's reproductive effort in the care of the male, it was expected that *N. acuminata* females would no longer exhibit aggression when removed from the vicinity of their eggs mass (their territory). The results show there was a significant difference in aggression exhibited between spent females (SS) compared to between sexually mature females (MM) (Figure 2.2). Although spent females have 'nothing to lose' as they have laid their eggs, no aggression was exhibited between spent females. Aggression was observed between spent and sexually mature females but aggression only reached level 1 and only in four out of ten trials. As females have already shed their eggs, unless a female is attempting to protect an egg mass, there will be no benefit to undertaking aggressive behaviour, as female death follows egg shedding (Reish, 1957). As females were removed from the vicinity of the parental tube and observed in a separate crystallising dish, the spent female may not exhibit aggression as there is no reason or benefit associated with such behaviour and no egg mass to protect from a potential competitor or predator. Great tit *Parus major* females have been found to be less aggressive towards the end of the breeding season (Slagsvold, 1993). However, this is contradicted by the theory that aggression should be increased as offspring get older and the probability of their survival increases (Jaroensutasinee and Jaroensutasinee, 2003). Female *N. acuminata* may have depleted energy levels or a decreased ability to show aggressive behaviour due to the shedding of eggs by dehiscence. Females lose a large proportion of their body mass through dehiscence (eggs fill the coelomic cavity as they develop), leaving a split in the structure of the body wall (Hardege, personal communication) with degeneration of musculature after dehiscence (Starczak, 1984). This change in the female may mean females are no longer capable of being aggressive or defensive, either due to the loss of body mass, lack of energy or due to the change in the body wall structure.

### 2.4.2: The effect of male aggression on pair formation

Female mate choice has been shown to be affected by male aggression (Forsgren, 1997; Doutrelant and McGregor, 2000; López *et al*., 2002). In some species, such as the shore crab *Carcinus maenas* (Van der Meeren, 1994), dominant males signal to females that they are of good quality, likely to be reproductively successful (Searcy, 1982). In other species, such as the sand goby *Pomatoschistus minutus* (Forsgren *et al*., 1996), dominant males are avoided, instead female choose males that indicate they are good fathers capable of caring for the female's offspring (Östlund and Ahnesjö, 1998). As *N. acuminata* females have been shown to prefer males capable of undertaking parental care, rather than aggressive males (Fletcher *et al*., 2009), the aggressive interaction was expected to increase the subsequent pairing times of males with females. The results showed no significant differences in mean pair times, with no effect of whether males were placed with familiar or unfamiliar females (Figure 2.3). Males and females were found to form pair bonds in less than 9 minutes in all cases. Aggressive bouts therefore in this case, do not influence pairing times, either with familiar or unfamiliar females. Only inexperienced males were used in the two trials and females could only pair form with the male that was presented to them, rather than given a choice between males. Pairs were formed in all replicates so there was no decision on the part of the female to reject a male. This could be due to female death following reproduction, if a male is not present when she sheds her eggs, it is unlikely that those eggs will be fertilised and produce viable offspring as eggs do not survive longer than approximately 2 days without paternal care (Reish, 1957).

There was a decrease in mean pair formation time with familiar females but this was not significantly different to the mean pair formation time observed when males were placed with unfamiliar females. It is possible that this decrease is due to the male and female recognising each other as former mates but as the difference in mean pairing times was not significantly different to the mean pairing time of males placed with unfamiliar females, there is no supporting evidence for this here. Performing more replicates may help to ascertain if mate recognition is occurring in *N. acuminata*. Separating pair-formed male and female individuals for varying amounts of time and comparing these interactions to those of the male and female when placed with unfamiliar potential mates, may help to determine if individual mate recognition occurs in this species.

With both trials, males were placed either with familiar or unfamiliar females, rather than placing one male with a familiar female and the other male with an unfamiliar female. It was therefore not possible in this experiment to see what effect the same aggressive interaction has on pairing, with either familiar and unfamiliar females and whether any differences are observed. Further to this, dominance and subordinate relationships were not examined between males, along with the effects of these relationships on subsequent female pairing. Dominant males have been shown to be preferred over subordinate males when females are presented with a choice of these two males (Fletcher *et al*., 2009); can the female detect this when she is not present in the fighting arena? Further to this, female presence may have an effect on male aggression. Although this was not examined here as aggression levels prior to males pairing with females were not taken, if males perceive a valuable resource such as a sexually mature female, this may alter the aggressive response (DiMarco and Hanlon, 1997). In *N. acuminata*, Fletcher *et al.* (2009) found that aggression levels were heightened between inexperienced males following pairing with a female. Aggression levels between two inexperienced males were also found to be heightened when a female was present in the experimental arena (Fletcher *et al*., 2009). It is possible that *N. acuminata* males may indicate to an opponent chemically that they have recently pair formed with a female, therefore affecting the aggressive response. In the velvet swimming crab *Necora puber*, exposing crabs to water conditioned using sexually receptive females led to prolonged aggressive interactions observed between two males, with more instances of behaviour seen with the potential to inflict injury (Smith *et al*., 1994).

### 2.4.3: The effect of previous aggression between males on subsequent interactions

Individual recognition for previous opponents has been reported in hermit crabs (Gherardi and Teidemann, 2004), lobsters (Karavanich and Atema, 1998; Katoh *et al*., 2008), swordtails (Franck and Robowski, 1987) and fish (Beacham and Newman, 1987). As fights can be both costly and pose a risk in terms of injury (Caldwell, 1985), it was expected that the aggression observed in subsequent fights between *N. acuminata* individuals would be less intense to reduce these effects. The results (Figure 2.4) showed that, although there was a decrease in the number of fights reaching level 2 and level 3 aggression over time, there was no significant difference in the aggression observed in subsequent fights between males. There was no evidence to suggest, therefore, that subsequent fights significantly alter the exhibited aggression between two individuals in this experiment. It is possible that individuals do recognise that they have fought previously but this does not have an effect of the aggression levels exhibited. Male aggression in the swordtail *Xiphophorus helleri* has been observed to increase with subsequent fights (Franck and Ribowski, 1987). It is therefore not clear from the experimental design that individual recognition for opponents is present or absent. Short-term learning has been reported in nereid polychaetes (Evans, 1966b) and is proposed to be slow (Evans, 1966c). In this experiment, individuals were isolated for two hours before individuals met again. It may be the case that this period of separation was too long and in this time, the recognition of individuals was lost. Further experiments should be conducted with individuals in each other's presence for longer than fifteen minutes using periods of separation that start small (such as one minute), building up to longer periods, to determine if there is an effect of individual recognition that was missed due to the experimental design used here.

### 2.4.4: Next steps

In the above experiments, aggression was examined in terms of the aggression level reached between two individuals. It may be the case that aggression levels remain relatively constant but the time engaged in fighting changes depending on the circumstances and the individuals placed together. New experimental designs should therefore be investigated, observing the time two individuals spend fighting, related to aggression levels recorded with each bout.

Female aggression was found to be absent when females where not in the presence of an egg mass following dehiscence. Females have been shown in a previous study to be aggressive when they have just laid eggs and are near to the parental male and the egg mass (Reish, 1957). Also, males have been shown to be aggressive when undertaking sole paternal egg care (Reish, 1957). Further experiments should be undertaken to determine if females (and males also) are aggressive towards intruding individuals of either sex prior to reproduction, when males and females are monogamously paired and sharing the same mucous tube.

It is also suggested that future studies should focus on individual recognition in terms of both fighting and pairing behaviour in this species to understand if males and females can recognise an individual that they have previously come into contact with. Winner and loser effects should also be examined in *N. acuminata* to determine if winners of fights also win subsequent fights (and losers lose subsequent fights) with unfamiliar individuals.

# Chapter Three:

## Intrasexual aggression between populations of *N. acuminata*

### 3.1: Experimental reasoning and aims

Aggressive interactions have been studied in a number of species and intrasexual aggression (between individuals of the same sex) may occur due to competition for resources such as space, food and mates or to defend territories or offspring (Clark, 1959). Both sexes may also engage in intrasexual aggressive interactions for social rank, dominance and potentially to prevent other individuals from mating or to try and be the first individual to mate with a particular partner (Karvonen *et al*., 2000). Such aggressive behaviour has been observed in the crabs *Necora puber* and *Liocarcinus depurator*, however female aggression was generally less intense than that of males (Huntingford *et al*., 1995).

Different species may occupy the same habitat and compete over resources. The two sympatric crayfishes *Cambarus bartonii bartonii* and *C. robustus* have asynchronous breeding and moult cycles and have largely non-overlapping distributions. In aggressive contests, *C. robustus* males were found to be dominant over males of *C. b. bartonii* when compared in size-matched fights (Guiasu and Dunham, 1999). In the hermit crabs *Clibanarius tricolour*, *C. antillensis* and *Calcinus tibicen* individuals all share a preference for gastropod shells and interspecific aggression has been observed over this limited resource used for predator protection (Bach *et al*., 1976). Similarly, in *C. antillensis* and *Pagurus crinitocarnis*, shell fighting is also likely and it has been found that *C. antillensis* individuals are generally dominant over *P. crinitocarnis* (Turra and Denadai, 2004). In the territorial ant *Formica pratensis*, aggression towards opponents decreases with increasing genetic relatedness, with more aggression therefore shown towards non-nestmates (Beye *et al*., 1998). This was also observed in the fire ant *Solenopsis invicta*, where individuals are also territorial and are highly aggressive towards members of other fire ant colonies compared to individuals of their own colony (Vander Meer and Alonso, 2002).

Same-sex aggression has been observed in many polychaete species, a few examples being *Platynereis dumerilii* (Evans, 1973), *Nereis virens* (Commito and Shrader, 1985) *Nereis pelagica* (Clark, 1959), *Nereis grubei* (Reish and Alosi, 1968) and also the experimental species *N. acuminata* (Reish, 1957). Individuals may use aggressive displays in order to defend territories, mates and offspring (Reish and Alosi, 1968; Evans, 1973). In *N. acuminata*, males are known to be aggressive towards any individual approaching the parental tube during egg care (Reish, 1957). Females that have shed their eggs are also known to be aggressive towards sexually mature females introduced in the vicinity of the parental tube where the male is caring for the recently laid egg mass (Reish, 1957).

Individuals can use assessment strategies to avoid fighting individuals that they are likely to lose against in order to lower the costs and risk of injury (Caldwell, 1985). Game theory predicts that individuals will compare their Resource Holding Potential (RHP), the ability of an individual to win a fight, to that of their opponents before deciding whether or not to escalate a fight (Sneddon *et al.*, 1997). The RHP of an individual can be influence by body size, age, sex (Beacham and Newman, 1987), morphology, physiology, previous aggressive experiences (Sneddon *et al.*, 1997), prior residency, energy reserves, resource value and physical condition (Morrell *et al.*, 2005). Asymmetries between opponents, where the RHP of each opponent differs, for example body size, can lead to the quick resolution of fights (Maynard Smith, 1974). Interactions between closely-matched individuals, however, are likely to escalate over time and increase the risk of injury to each individual (Maynard Smith, 1974). The fight will continue until the individual with the lowest cost threshold reaches that level (e.g. expended energy levels) meaning that the duration of the fight is determined by the RHP of the individual with the lower cost threshold, the eventual loser (Morrell *et al.*, 2005).

In order to decrease the risk of injury incurred during combat, communication systems capable of transmitting sufficient information to the opponent would be expected to evolve if weapons used in aggressive interactions are potentially lethal (Caldwell, 1979). In *N. acuminata*, aggression potentially involves such behaviour as the jaws are used in fights to attack the opponent, which can escalate to severe aggression involving biting (Reish and Alosi, 1968). Communication signals may be shaped by features of the biotic and abiotic environment and differences in habitat conditions occupied by populations can affect signalling systems (Lovern *et al.*, 1999). Closely related species often exhibit great signal diversity and such divergence in communication signals is a factor that can promote speciation (Leal and Fleishman, 2004). Signal diversity may evolve because species or populations occupy different habitat conditions where selection for effective communication promotes divergence in signal design. This may occur due to differences in habitat condition which favour differences in sensory systems which in turn select for differences in signal design (Leal and Fleishman, 2004). Alternatively, differences in habitat noise and transmission properties may favour differences in signal design even if there is no change in the sensory system

(Leal and Fleishman, 2004). If there is a change in the signal design, reproductive isolation may occur due to a failure to communicate if such signals are important for mate choice or species recognition (Leal and Fleishman, 2004). Divergence of populations can also be affected by the level of predation, parasite prevalence (Madden, 2006), food availability and/or density (Ward and McLennan, 2008). If local or geographical selective regimes are different and strong enough, behaviour in different populations of a species may evolve in different directions (Lahti *et al.*, 2001). Variation in traits such as metabolic rates, trophic structures, acclimatisation abilities, thermal and osmotic tolerances and life history parameters can also be expressed through changes in behaviour (Ptacek and Travis, 1996).

Divergence between populations may therefore be expressed in changes in behaviour, including aggression. Dunbrack and Clarke (2003) stated that the levels of aggression in pair-wise contests should be higher the greater the behavioural divergence there is between two opponents. Individuals from different populations should display divergence that is greater than that shown between two individuals from the same population. This is known as the communication failure hypothesis and can lead to the escalation of fights between individuals (Maynard Smith and Riechert, 1984). As mentioned before, asymmetries in RHP are likely to lead to the quick resolution of fights from an initial assessment of the opponent (Maynard Smith, 1974) and escalated fights are more likely when cues from the initial assessment are ambiguous and individuals must use more overtly aggressive behaviours in order to determine the outcome of the fight (Maynard Smith, 1974). Communication signals which deviate from the population norm could be sufficiently ambiguous that it provokes a more aggressive response (Dunbrack and Clarke, 2003). Evolutionary modifications of communication signals may lead to divergences in population behaviour, causing higher levels of aggression or prolonged fights between populations in pair-wise contests due to a mutual ambiguity in individual assessment (Dunbrack and Clarke, 2003). Population comparisons are therefore used to gain insight into the causes of behavioural differentiation (Lahti *et al.*, 2001) and the selective influence of the environment on signal expression (Lovern *et al.*, 1999).

Previous studies have shown behavioural divergences within populations (Saito, 1995; Lahti *et al.*, 2001). Aggression levels were examined within population of the brown trout *Salmo trutta* (sea-run, lake-run and resident populations) with migratory populations exhibiting higher aggression levels when compared to the aggression observed within the other populations (Lahti *et al*., 2001). Among populations of the spider mite *Schizotetranychus miscanthi*, there was found to be high variations in the aggressive behaviour with mean winter temperature of the sample site positively correlated with the level of aggressive exhibited (Saito, 1995). Aggression has also been examined in staged contest between individuals from different populations by Dunbrack and Clarke (2003). In their study, two populations from different riverine systems of the brook trout *Salvelinus fontialis* were compared in size-matched contests, using nipping rate as the index of aggressiveness. The mean number of nips in fights between individuals from different populations was found to be greater than the mean number of nips in fights involving individuals from the same population, showing escalated fights between individuals from different populations (Dunbrack and Clarke, 2003).

As *N. acuminata* populations were sampled from the wild and from different types of habitats, differences in the environment may have led to a divergence in behaviour. The Reish (R) and Los Angeles (LA) populations were sampled from LA Harbour (R collected in 1964, LA in 2008), the Newport (N) population was collected from Newport Beach (in 2005), the San Gabriel (SG) population was collected from San Gabriel River (in 2003) and the Connecticut (C) population was collected from Alewife Cove (classed as an estuary, collected in 2002). Genetic divergence in the four laboratory populations has already been investigated (Pesch and Pesch 1980; Weinberg *et al*., 1990; D.H. Lunt and J.D. Hardege, unpublished). Not only is there a difference in the chromosome number of the C population (2n = 22 in the C population compared to 2n = 18 in R, N and SG populations) (Pesch and Pesch 1980; Weinberg *et al*., 1990) the C population, according to the nuclear DNA, is likely to have diverged from the other three populations approximately ten million years ago. Examining the mitochondrial DNA, following the divergence of the C population, the SG population diverged from the R and N populations approximately seven million years ago. R and N have been found, using the mitochondrial DNA, to be very similar genetically, with little divergence (D.H. Lunt and J.D. Hardege, unpublished).

Previous work on the aggressive relationships between populations of *N. acuminata* has not examined the intrasexual aggressive behaviour. Therefore, the levels of intrasexual aggression both within and between the four laboratory populations were examined. Aggression between males was examined, as was the aggression between females. Differing aggression levels may add supporting evidence to show divergence of the populations of *N. acuminata*. It was expected that individuals from different populations would exhibit higher aggression levels towards each other than individuals from the same population, with both males and females. As the populations all share the same life history traits and are very similar morphologically (Weinberg *et al*., 1990), aggression levels may be used to identify the separate groups and the divergence between the populations may be expressed via heightened aggression due to the communication failure hypothesis, indicating that escalated aggression would be expected between populations that have diverged (Dunbrack and Clarke, 2003).

In addition, the new wild Los Angeles (LA) population (sampled in 2008) was compared to the existing laboratory maintained Reish (R) population with both males and females to determine if there are any differences in aggression between these two populations and whether these results suggest that these two populations should be treated as potentially separate species. Studies undertaken by Weinberg *et al*. (1990; 1992) have caused some controversy as it has been suggested that the R population had speciated in the laboratory since its collection in 1964. Weinberg *et al*. (1990) concluded that due to high levels of pre-mating isolation between the R population with both SG and N populations compared to low levels of pre-mating isolation between SG and N, speciation occurred in the R population in the laboratory following a founder event. Weinberg *et al*. (1992) subsequently looked at the percentage of broods that produced healthy offspring between the R, SG and N populations (a post-mating isolation experiment). When R individuals were paired with either SG or N individuals, no healthy offspring were produced. However, when SG individuals were paired with N individuals, the percentage of healthy offspring was found to be 77% (normal levels were calculated as 75-95%). These controversial findings were therefore further discussed in the work by Rodriquez-Trelles *et al*. (1996). This work disagreed with Weinberg *et al*. (1992) and hypothesised that the two wild populations used in the study (SG and N) were not representative of the population from which the R population was originally sampled from. The study went on to investigate the genetic markers, genetic variability and divergence of the three populations. It was found that in 13/18 loci, the R population does not share any alleles with either SG or N and high genetic differences (D) were found to be as follows: SG to N = 0; R to SG = 1.75; R to N = 1.76. The genetic difference between the two sibling species *N. diversicolor*, a population from Germany, and *N. limnicola*, a species from the U.S.A., is 1.28. The study therefore concludes that these three populations formed separate species before the R population was sampled in 1964. As the R population and the wild LA populations were collected from the same geographical location, the LA population may provide a better representation of the population that R was originally sampled from. Due to changes in the environmental conditions in Los Angeles Harbour due to the pollution abatement programme (Reish *et al.*, 1980) and as the R population has been maintained in the laboratory under constant light, temperature and feeding regimes, it is hypothesised that these two populations will have diverged. As these populations have been separated for 44 years, it is hypothesised that aggression levels between these two populations would be higher than the aggression within each population.

In nature, the male is generally the more aggressive sex due to its relationship with mating and mating success, where females are often choosy and males actively court such females, undertaking 'traditional' sex-roles (Berglund *et al.*, 1986). However, female competition and male mate choice (sex-role reversal) has been observed to occur when the sex ratio is female-biased (Côte and Hunte, 1989), if mating is more costly for males (Ridley, 1978) or when males contribute greatly towards parental investment (Gwynne, 1981). In the case of the experimental species *N. acuminata*, Starczak (1984) observed that winning fights when an individual of the opposite sex was present was more prevalent when two males were placed with a female compared to two females placed with a male. Males are also typically the more aggressive sex if there is limited availability of females (Kvarnemo *et al.*, 1995). Due to female death following egg release in *N. acuminata* and because more monogamous breeding events occur over time, it is likely that the sex ratio in a population will become male-biased (Starczak, 1984). Males are also able to reproduce again once the eggs have hatched and larvae have left the parental tube (Reish, 1957). The final aim of this chapter was to compare the levels of aggression found between male individuals to those found between females. As female choice and male aggression for females is evident in this species (Starczak, 1984; Fletcher *et* al., 2009), it was hypothesised that female aggression intensity will be lower than that of males. Therefore, levels of aggression were compared to determine the sex displaying the highest levels of aggression to see if this provided further indication of the occurrence of female choice in *N. acuminata*.

## 3.2: Methodology

Individuals used in the following experiments were selected at random from the stock population tanks maintained at the University of Hull, each containing over 100 adult individuals and only sexually mature worms were used. Males and females were identified by the colouration of the gametes in the coelomic cavity: white for males and yellow for females. Any ambiguity in coloration was resolved by placing the individual with another and observing the behaviour for a short space of time to determine if pairing occurred. The four laboratory populations plus the recently sampled wild population, detailed in section 1.10, were used: Reish (R), Newport Beach (N), San Gabriel River (SG), Connecticut (C) and Los Angeles Harbour (LA). Individuals used in experiments were size-matched to within approximately a 2mm range of each other to eliminate the possibility that size may influence aggressive interactions. Sexually mature individuals typically measure approximately 60mm in length.

Two sexually mature males were selected from the designated population aquaria and placed in a crystallising dish (70mm diameter) containing 40 ml of sea water (salinity 36-38‰). The salinity in each of the population tanks ranges between 35-40‰. Crystallising dishes of this size were used to allow individuals to interact but also to separate following any aggressive interactions to minimise the risk of injury. Males were distinguished from each other by observing the faecal contents in the gut which vary between individuals due to differing feeding habits, giving a unique ‘pattern’ (Fletcher *et al*., 2009). Individuals were used only once in any aggressive interaction. The level of aggression was observed between the two individuals, as detailed in Figure 2.1 (page 45) over a fifteen minute period and scored as follows:

Level 0: no aggression between the two individuals.
Level 1: individuals avoid contact with each other, palpi may be slightly flared.
Level 2: a fighting position is assumed by both individuals with the palpi flared and the jaws visible.
Level 3: severe aggression is observed with biting of the opponent.

The experiment was initiated following frontal contact between the two individuals. Therefore, the aggression score is not a score of individual aggression but of the interaction between the two individuals. As previous studies have only looked at observations over a 5 minute time frame (Reish and Alosi, 1968; Fletcher *et al*., 2009), the 15 minute observation period was used to provide a good indication of the aggression, if any, displayed between individuals.

For each replicate, the highest aggression level overall (0, 1, 2 or 3) was recorded to provide an accurate level of intensity for each interaction and to avoid any effects of continuous fighting or an escalation of fights on the aggression recorded.  Aggression was observed between each of the different population groups, with n = 20 pairs of individuals.

Finally, aggression was observed between individuals from the wild LA population (n = 20 pairs) and these were compared to the aggression observed between individuals from the LA population and individuals from the laboratory-based R population (n = 20 pairs) using the same method as described above.  The aggression found within the R population as described above (n = 20 pairs) was also used as a comparison to the aggression observed between LA and R individuals.  The above protocol was then repeated using two sexually mature females, with aggression level assessed again using the different population groups.  The total number of interactions observed was 440.

## 3.3: Results

### 3.3.1: Male aggression within and between the four laboratory populations

Within each of the four laboratory populations, the majority of the aggressive interactions only reached level 1 and interactions were found to have low frequencies of the highest level of aggression (level 3), involving severe attacks with biting, as Figure 9 below demonstrates. Aggression level frequencies observed for each combination of males were compared to determine if there were any differences in aggression levels between individuals originating from different populations. Figure 3.1 below shows the number of fights reaching each level of aggression when combinations of males from each of the populations were placed together. There was variation in the number of fights reaching each level of aggression depending on the individuals that were placed together. The aggression levels recorded for each combination of individual were compared using the Kruskal-Wallis test.

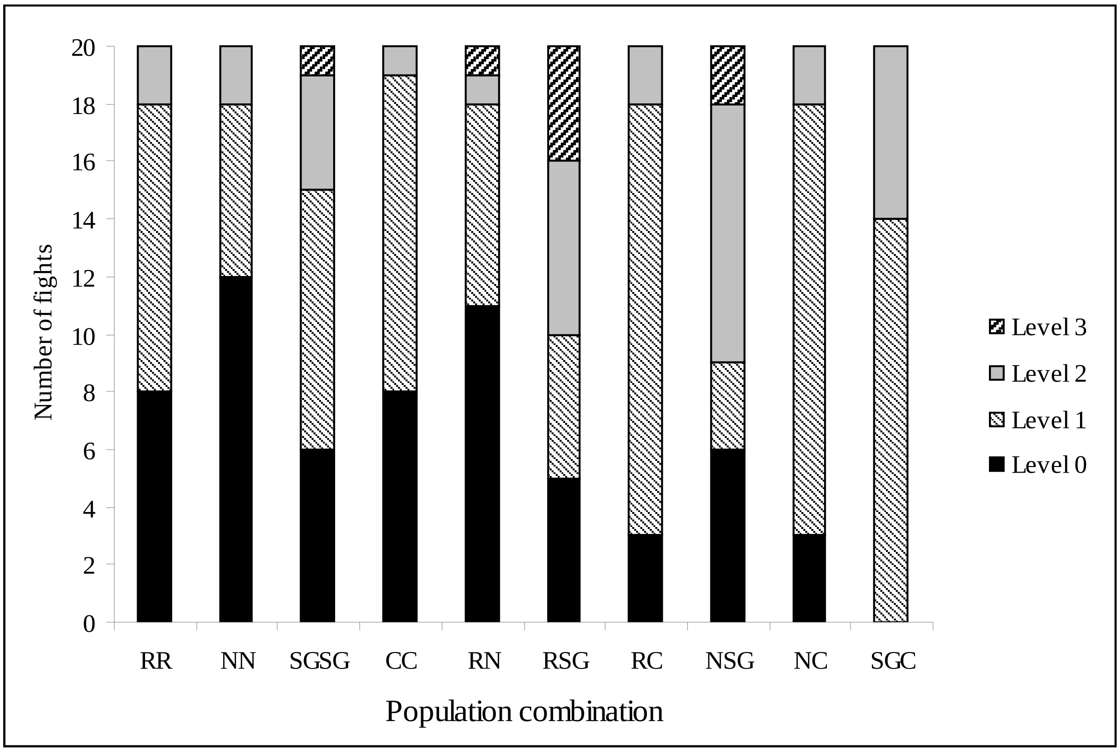


Figure 3.1: The number of fights reaching each level of aggression for each combination of males, with comparisons for each of the four populations: Reish (R), Newport (N), San Gabriel (SG) and Connecticut (C). Each bar represents 20 replicates.

There was a significant difference found when the aggression levels were analysed using the Kruskal Wallis test ($\chi^2/_2$ = 30.6376, P = 0.0002). A post-hoc Nemenyi-Damico-Wolfe-Dunn test, according to Zar (1996), was then performed in the statistical package R (version 2.10.1) to determine which groups differed. Significant differences in aggression level were found between NN and NSG (P = 0.0346), between NN and RSG (P = 0.0193), between NN and SGC (P = 0.0114) and between SGC and RN (P = 0.0343). No other groups displayed significant differences in aggression (P > 0.05).

### 3.3.2: Male aggression between the laboratory (R) and the wild (LA) population

Observed aggression frequencies for each combination of the R and LA populations are demonstrated in Figure 3.2 below. The recorded aggression was analysed using the Kruskal Wallis test. There were no significant differences between the aggression levels from RR, RLA and LALA ($\chi^2/_2$ = 2.721, d.f. = 2, P = 0.257). Although the RR aggression data has been analysed statistically in the previous section, Rice (1989) suggests that a correction need only be made when two or more tests that cannot be pooled attempt to answer the same null hypothesis. As this data set is examining the differences in aggression level to the LA population and not the populations compared previously, a different hypothesis is being tested here.

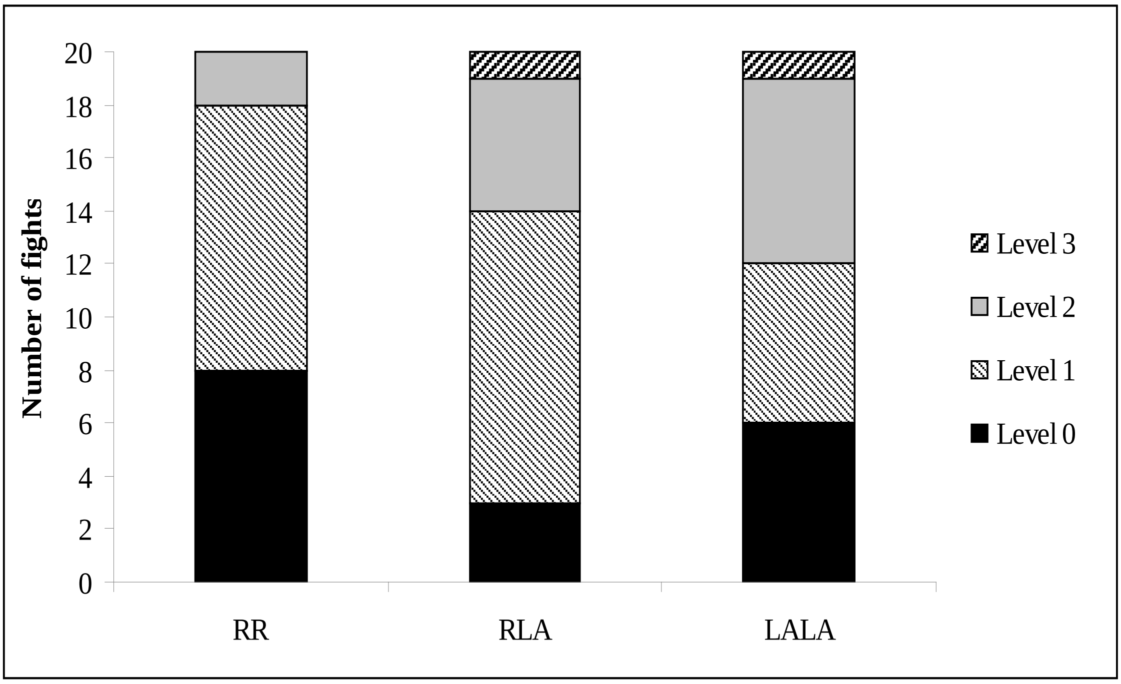


Figure 3.2: The number of fights reaching each level of aggression with interactions within the laboratory Reish population (RR), between the Reish population and the wild Los Angeles population (RLA) and within the wild Los Angeles population (LALA). Each bar represents 20 replicates.

### 3.3.3: Female aggression within and between the four laboratory populations

Within the R, N, SG and C laboratory populations, the majority of the aggressive interactions only reached level 1 and interactions were found to have low frequencies of the highest level of aggression (level 3); fights were found to have low levels of aggression involving severe attacks with biting, as Figure 3.3 below demonstrates. Aggression level frequencies observed within each of the four laboratory populations were compared to the frequencies found when members of the different populations were placed together to determine if there were any differences in aggression levels between individuals originating from different populations. Figure 3.3 below also shows the number of fights reaching each level of aggression when combinations of females from each of the populations were placed together. There was variation in the number of fights reaching each level of aggression. The aggression levels recorded for each combination of individual were compared using the Kruskal-Wallis test, showing there was no significant difference in the aggression levels observed between each combination ($\chi^2/_2 = 12.523$, d.f. = 9, P = 0.185).

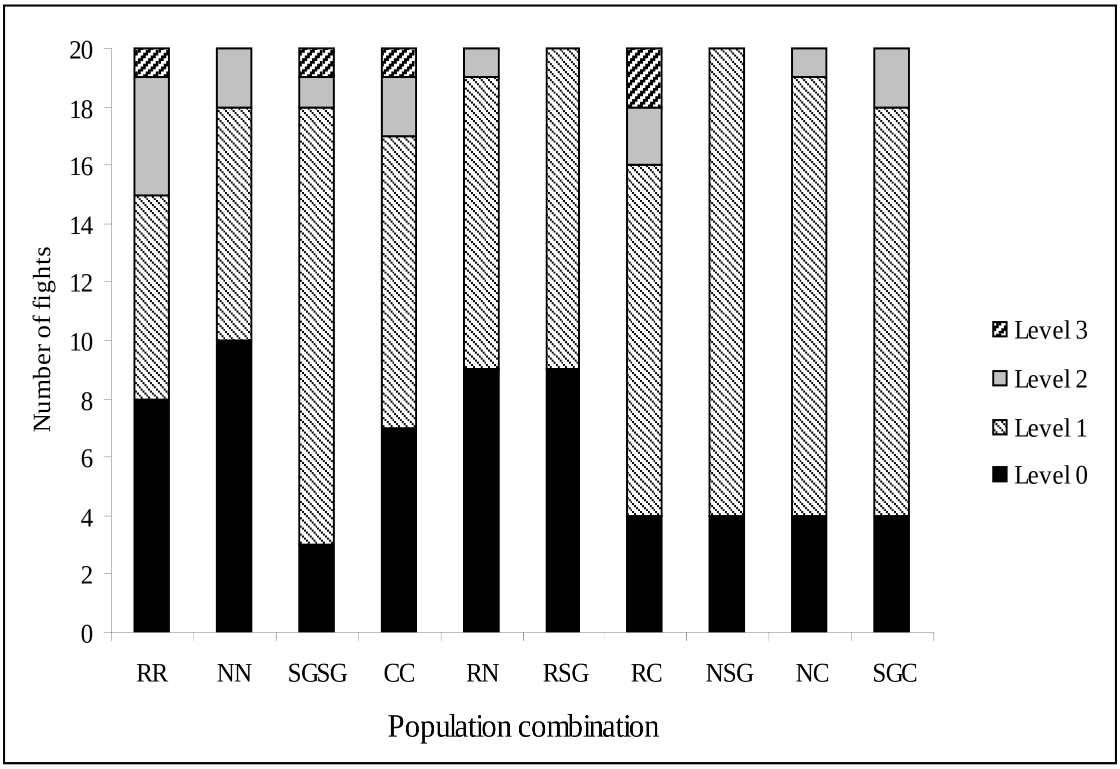


Figure 3.3: The number of fights reaching each level of aggression for each combination of females, with comparisons for each of the four populations: Reish (R), Newport (N), San Gabriel (SG) and Connecticut (C). Each bar represents 20 replicates.

### 3.3.4: Female aggression between the laboratory (R) and the wild (LA) population

Observed aggression frequencies for each combination of the R and LA populations are demonstrated in Figure 3.4 below. The recorded aggression was analysed using the

Kruskal Wallis test and there significant differences were found ($\chi^2/_2$ = 8.832, d.f. = 2, P = 0.012). A post-hoc Nemenyi-Damico-Wolfe-Dunn test, according to Zar (1996), was then performed to determine which groups differed. A significant difference in aggression level was found between RR aggression and RLA aggression ($q_{0.05,\infty,3}$ = 3.314, q = 3.969) but not between either RR and LALA ($q_{0.05,\infty,3}$ = 3.314, q = 2.561) or between RLA and LALA ($q_{0.05,\infty,3}$ = 3.314, q = 1.408). Although the RR aggression data has been analysed statistically in the previous section, Rice (1989) suggests that a correction need only be made when two or more tests that cannot be pooled attempt to answer the same null hypothesis. As this data set is examining the differences in aggression level to the LA population and not the populations compared previously, a different hypothesis is being tested here.

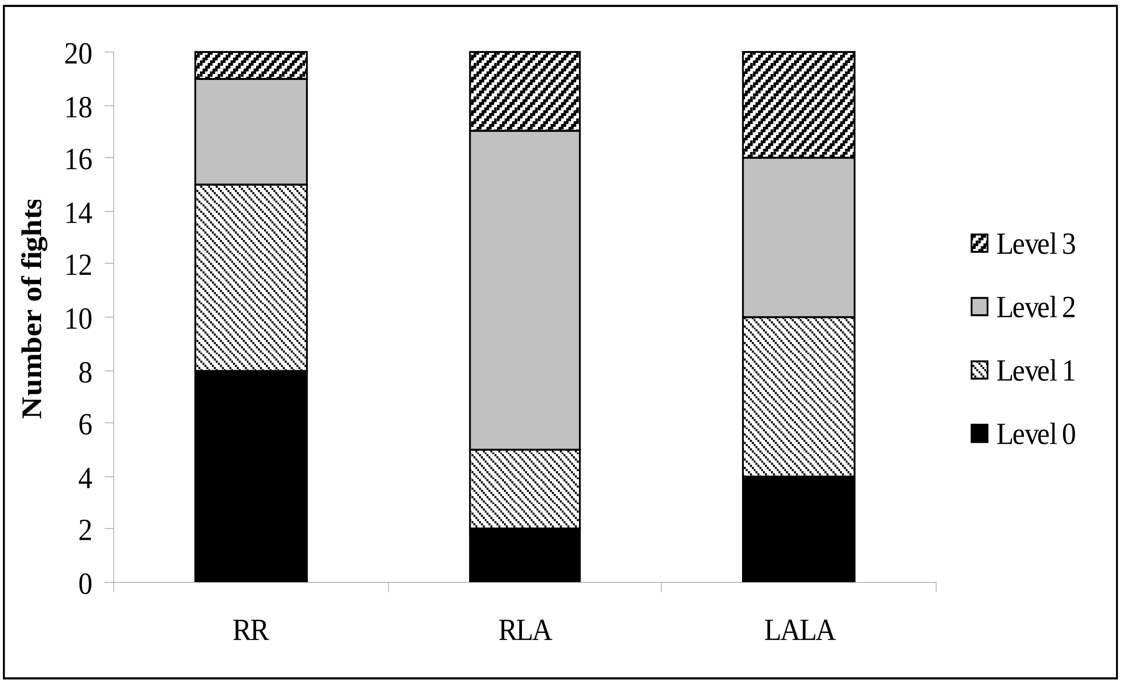


Figure 3.4: The number of fights between females reaching each level of aggression with interactions within the laboratory Reish population (RR), between the Reish population and the wild Los Angeles population (RLA) and within the wild Los Angeles population (LALA). Each bar represents 20 replicates.

### 3.3.5: Male aggression compared to female aggression within each population

The results indicate that in most cases, frequencies of female aggression were found to be similar to the frequencies of male aggression, as displayed in Figure 3.5 below.

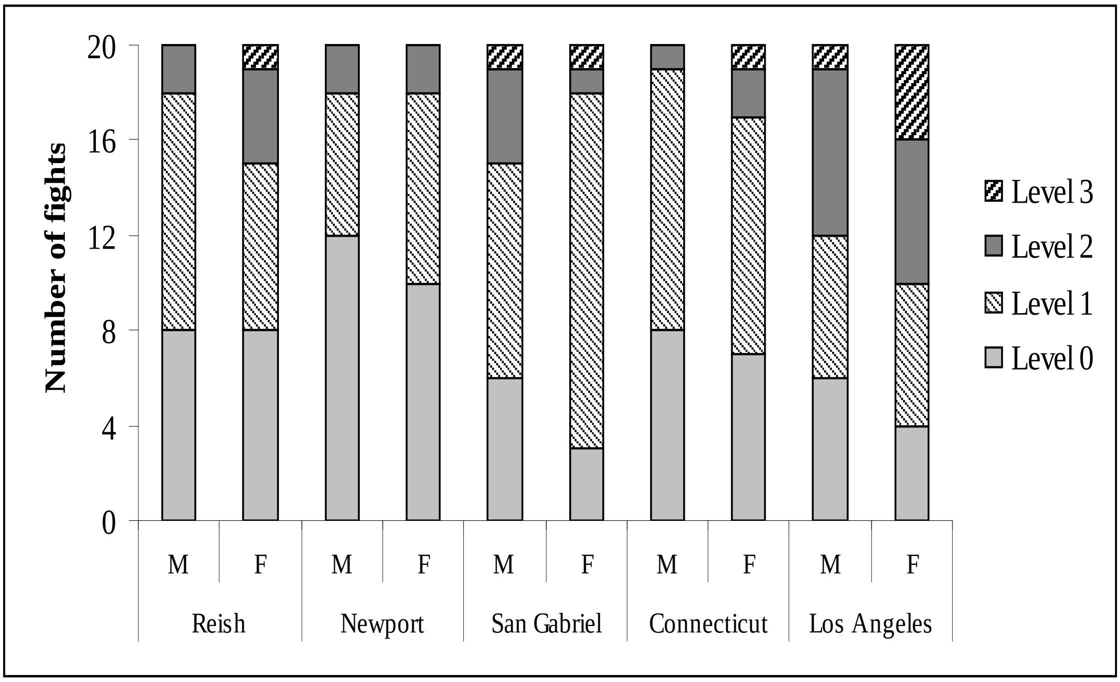


Figure 3.5: The number of fights reaching each level of aggression between males (M) and females (F) from each of the populations, Reish (R), Newport (N), San Gabriel (SG), Connecticut (C) and Los Angeles (LA).

With all five populations, the number of fights showing no aggression was greater in males than females. Observing interactions within the Reish population, the number of fights reaching level 1 was higher between males. Females had more fights reaching levels 2 and 3 than males. With the Newport population, the number of fights reaching level 1 was higher in females, level 2 was higher in males and the number of fights reaching level 3 was equal. With the San Gabriel population, the number of fights reaching level 1 was higher in females, level 2 was higher in males and equal for fights reaching level 3. With the Connecticut population, fights reaching level 1 was higher between males. Females had more fights reaching levels 2 and 3 than males. With the Los Angeles population, the number of fights reaching level 1 was equal. The number of fights reaching level 2 was higher in males whereas the number of fights reaching level 3 was higher in females.

For each of the 5 populations R, N, SG, C and LA, differences in male aggression were compared to differences in female aggression using the Mann Whitney U test. As detailed in Table 3.1, there were no statistical differences observed between the aggressive frequencies of males to females in any of the populations. Again, although the aggression recorded for each of there populations, both male and female, have been analysed statistically before, this analysis is testing a different hypothesis comparing male and female aggression. As previously mentioned, Rice (1989) suggests that a correction need only be made when two or more tests that cannot be pooled are used to attempt to address the same null hypothesis.

Table 3.1: Statistical results comparing observed male aggression to female aggression for each population using the Mann Whitney U test.

| Population | U value | P value |
|---|---|---|
| R | -0.555 | 0.579 |
| N | -0.548 | 0.584 |
| SG | -0.092 | 0.926 |
| C | -0.664 | 0.507 |
| LA | -1.043 | 0.297 |

## 3.4: Discussion

### 3.4.1: Aggression within and between the four laboratory populations

Game theory predicts that individuals should compare their RHP with that of their opponent before escalating aggression both before and during a fight (Sneddon *et al*., 1997). Contests are predicted to be resolved more quickly the more the strengths and weaknesses of the opponents differ, known as asymmetries between contestants (Gammell and Hardy, 2003). Where opponents are evenly-matched in RHP, fights are likely to escalate and increase the risk of injury to each opponent (Maynard Smith, 1974). According to the communication failure hypothesis, fights are also likely to be escalated if there is behavioural divergence between the two opponents due to the expression of communication signals which deviate from the norm (Dunbrack and Clarke, 2003). Individuals from different populations should display divergence that is greater than that shown between two individuals from the same population (Dunbrack and Clarke, 2003). Differences in aggression can be exhibited when comparing levels of aggression within a population to other populations in different habitat condition, such as the variation in aggression levels observed within different populations of the brown trout *Salmo trutta* (Lahti *et al*., 2001). Differences can also be exhibited in staged contests between individuals from different populations, as observed in size-matched contests of individuals from two populations of the brook trout *Salvelinus fontialis* (Dunbrack and Clarke, 2003).

The aggression of both males and females within the four populations with R, N SG and C were found to be similar. The male aggression results (Figure 3.1) showed that within each of the four laboratory populations (R, SG, N and C), the majority of fights only reached level 1 aggression and fights were found to have low occurrences of level 3 aggression, involving severe attacks with biting. Similarly, the female aggression results (Figure 3.3) showed that within each of the populations N, SG and C, the majority of fights only reached level 1 aggression. Within the R population, there were more fights that reached level 2. Again, fights were found to have low occurrences of level 3 aggression. With both male and female aggression within each of the populations, as the aggression level increased, the frequency of fights reaching that level decreased within all four populations. The low level of aggression found may be due to assessment of opponents, with ritualised displays of aggression without incurring injury rather than escalated aggression between opponents. Due to the eversion of the jaw and biting exhibited with level 3 aggression (Reish and Alosi, 1968), individuals may not engage in this type of aggression to avoid a bite from the opponent. It could be the case in *N. acuminata,* that within populations, individuals assess the RHP of their opponents and resolve fights quickly to avoid risky and costly fighting behaviour. There were no significant differences found in female aggression with any of the four populations R, N, SG or C. Significant differences were found in the male aggression results however but the post-hoc test showed that there were no significant differences when comparing aggression found within each of the four populations (comparing RR, NN, SGSG and CC). Therefore, if divergence of these populations does cause a change in behaviour, it is not exhibited through aggression between individuals from the same population.

Individuals in these experiments were size-matched to eliminate size as a variable in the aggression displayed between two individuals. As *N. acuminata* individuals are typically difficult to measure, opponents were size-matched to within 5mm, where sexually mature individuals measure approximately 60mm (Pettibone, 1963). As previously mentioned, symmetries in RHP can lead to escalated fights (Maynard Smith, 1974). It is possible that the symmetry in body size used in these experiments with matching opponents has led to individuals exhibiting a greater level of aggression towards each other. It is also possible that although worms were size-matched to their opponents, there were differences in the size ranges of individuals between the replicates, with some individual fights involving smaller individuals than other fights.

Morrell *et al*. (2005) found that in the fiddler crab *Uca mjoebegi*, fight duration increased with increases in the mean size of closely-matched opponents. This suggests that larger individuals are capable of fighting for longer periods than smaller opponents (Morrell *et al*., 2005). It is possible in *N. acuminata* that fights involving larger individuals could have impacted upon the results. However, sexually mature individuals were used in all replicates. In females, only sexually mature individuals were used, meaning that egg were visible in the coelomic cavity, excluding females that are smaller. Females do not generally grow bigger than 60mm, as eggs fill the cavity, individuals cease feeding, preventing further growth (Reish, 1957). In males, only sexually mature individuals were used as opposed to a male that had reproduced already as experienced males are older and can be larger than inexperienced individuals. Therefore, any size discrepancies would have been relatively small between the replicates and attempts were made to control the age of the individuals used so individuals larger than approximately 60mm were not used.

The male aggression results show that there were significant differences in aggression between the combinations of opponents from the four populations (Figure 3.1). The post-hoc test showed however that there were only significant differences between aggression when comparing NN aggression to NSG aggression, NN to RSG aggression, NN to SGC aggression and SGC and RN aggression. With female aggression, however, although aggression within and between the four populations was also found to vary (Figure 3.3), there were no significant differences found in aggression between any of the combinations of females placed together. According to the communication failure hypothesis, escalation of fights can occur if during the initial assessment of an opponent, the information received is ambiguous (Dunbrack and Clarke, 2003). This could explain why there are significant differences in aggression recorded, if between certain individuals, fights are escalated and reach a higher level of aggression involving serious attack and biting. However, if this were the case, female aggression would also be expected to differ significantly from aggression involving individuals from the same population and this was not observed.

Males appear to be more aggressive towards individuals from other populations, as demonstrated by the significant differences in aggression observed. It could be that female aggression towards individuals from other populations is naturally less intense than male aggression. Female aggression has been observed to be less intense than male aggression in the American lobster *Homarus americanus* (Peeke *et al.*, 1998). The results indicate that females exhibit the same intensity of aggression towards an individual, regardless of the opponent's population. Females may not be able to recognise each other as originating from different populations and therefore exhibit the same level of aggression to all females presented.

### 3.4.2: Aggression between the laboratory (R) and the wild (LA) population

The male aggression results between the R and the LA populations showed no significant differences (Figure 3.2), either within or between the two populations. This suggests that individuals may not recognise each other as different. Although these two populations have been separated for 44 years, the R population may still form a representative population that is comparable to the population found in natural conditions, such as the LA population.

With female aggression however, a significant difference was observed when the aggression within the R population was compared to that between the R and LA populations (Figure 3.4). The aggression between the other combinations was found to have no significant differences. The aggression between R and LA individuals was found to have higher incidences of level 2 aggression compared to aggression from within both R and LA. It may be the case that females are recognising a signal that the males are not, although the results for the other four population combinations contradict this as female aggression was not different, whichever females were placed together. These two populations have been separated with one population reproducing in the laboratory and the other in the wild for approximately 44 years so this difference in environmental conditions could be attributed to this. As previously mentioned, differences in habitat conditions such as food availability, predator abundance, light intensity and temperature can alter the communication system (Lovern *et al.*, 1999) and cause an escalation of fights due to ambiguity in the initial assessment of opponents (Dunbrack and Clarke, 2003). The R population was collected in 1964, prior to the pollution abatement programme of Los Angeles Harbour that was introduced in 1968 (Reish *et al.*, 1980). The R population has subsequently been maintained under laboratory conditions, with constant light, food and temperature regimes. The LA population was collected in 2008, after a period of dramatic change in the harbour including the pollution abatement and an oil tanker explosion (Reish, 1986). These differences in environmental conditions between the two populations could have caused changes in the signal design and/or the sensory systems in *N. acuminata*.

However, it seems unlikely that changes in the signal design or sensory systems, leading to changes in aggression, would be expressed in one gender and not the other. Female aggression between the R and LA populations was not found to be significantly different to LALA aggression, only to RR aggression. It is possible that this is due to variation in aggression exhibited between individuals. Aggressive behaviour has been described as being genetically complex with loci sensitive to the physical and social environment, potentially causing deviations in behaviour (Edwards *et al.*, 2009). It is possible in *N. acuminata* that slight variations between individuals in their genetic composition could cause deviations in aggressive behaviour. More replicates should therefore be undertaken to determine the extent of variation in aggression between the R and LA populations.

The aggression results found in this chapter within and between populations of *N. acuminata* were difficult to interpret. It is unclear why there would be heightened aggression in males and not females for the combinations of R, N, SG and C populations, but reversed with heightened female aggression but not male aggression when comparing the aggression for the combinations of the R and LA populations. It is possible that with further replicates, these differences would become clear, either in the case of male aggression, or female aggression.

### 3.4.3: Male aggression compared to female aggression within each population

There were no significant differences found when male aggression was compared to female aggression for each of the populations, R, N, SG, C and LA (Figure 3.5). In previous studies, higher levels of aggression in males were linked to the incidence of sexual selection in this species. As males display aggressively and females are choosy, aggression in females will not be linked to reproduction but is more likely to exist for competition for resources, for example food and space (Clark, 1959). This is supported in the study by Starczak (1984) that found that females were not interested in fighting. Aside from defending territory or resources, there may be no other benefit to the female exhibiting aggression. In pipefish species, male-to-male competition has also been found to be stronger than female-to-female competition suggesting the sex roles are not reversed (Matsumoto and Yanagisawa, 2001).

As there were no significant differences between male and female aggression, the results do not provide support for the argument for female mate choice and male aggression in this. However, aggression levels in this study were only examined by placing two members of the same sex together. Starczak (1984) found that aggression levels between two males were higher but only when a female was present. As no member of the opposite sex was present during the aggressive interactions, the aggression levels recorded may not be related to reproduction, instead showing aggression due to lack of food or space, for example.

In summary, the evidence suggests that although male individuals react differently when placed with an individual from another population, aggression is a weak indicator of population divergence. The R population may still be a representative sample of the wild population LA, even though these two populations have been separated by approximately 44 years.

### 3.4.4: Next steps

Further work on aggression in this species needs to be undertaken including studies looking at aggression from an individual rather than observing the aggressive interaction between two individuals. It may be the case that one individual shows aggression and the opponent responds in turn to this aggression display. It would be interesting to determine if one population started the aggressive bout over another. Further to this, work needs to be undertaken looking at the potential resources that individuals may fight over, for example mates, food and assessing any differences between male and female needs for particular resources. Experimental work could also be undertaken to look at the population water for this species, observing if placing an individual in the water from a different population affects the subsequent aggression exhibited towards a member from that population. Molecular work on the Los Angeles population also needs to be undertaken to determine the genetic relationship of this population to the other populations maintained in the laboratory.

The pairing behaviour between these four laboratory populations (R, N, SG and C) and the LA wild population was examined further in Chapter 4.

# Chapter Four:

## Pair formation and pre-mating isolation between populations of *Nereis acuminata*

### 4.1: Experimental reasoning and aims

As mentioned in Chapter 3, the diploid chromosome numbers of the different populations was found to be 18 in the Reish (R) population (Pesch and Pesch, 1980), 18 in the San Gabriel (SG) and the Newport (N) populations but 22 in the Connecticut (C) population (Weinberg *et al.*, 1990). Chromosome numbers were also documented in other nereid polychaetes and are as follows: *Nereis limbata* diploid 20-30, *Platynereis megalops* haploid 14, *Nereis diversicolor* diploid 32 and *Perinereis cultrifera* diploid 34 (Pesch and Pesch, 1980). Differences in chromosome number can be used as indicators of reproductive incompatibility (Knowlton, 1993). Changes in chromosomes can play a role in evolutionary change, including the loss or gain of whole chromosomes, but this may not influence any morphological change in a species (John, 1981). Such chromosome change can, but does not always, influence speciation and act to isolate individuals reproductively (John, 1981).

The pairing behaviour both within and between populations of *N. acuminata*, including both pre- and post-mating isolation trials, has been examined in order to determine levels of reproductive isolation (Weinberg *et al.*, 1990; Rodriquez-Trelles *et al.*, 1996; Sutton *et al.*, 2005). Reproductive isolation is known as the final step before speciation and individuals can either be isolated by pre-zygotic barriers or by post-zygotic barriers (Snell, 1989). Pre-zygotic barriers include differences in behaviour, mating preferences (Snell and Hawkinson, 1983), mating season, fertilisation mechanisms (Snell, 1989) habitat and spawning synchrony (Palumbi, 1994). Post-zygotic barriers include genetic incompatibility, hybrid sterility and hybrids that are not viable (Snell, 1989).

Pre-mating bioassays are generally simple and quick to undertake and are useful for sibling species where morphological differences may be minimal (Snell, 1989). In the marine copepod *Acartia clausi*, the west and east coast populations from the U.S.A. are morphologically similar but have diverged and are reproductively isolated (Carrillo *et al.*, 1974). In the experimental species *N. acuminata*, pre-mating isolation trials are suitable to use due to the initial pairing behaviour of a male and a female before the eggs are shed by the female. This behaviour is easy to observe and gives a good indication of compatibility. Weinberg *et al.* (1990) looked at the pre-mating isolation levels over the course of ten minutes using two populations from the Atlantic Ocean: Massachusetts (M) and Connecticut (C), and two from the Pacific Ocean: San Gabriel (SG) and Newport (N). Pairing behaviour was not observed between male and female individuals from the Atlantic and the Pacific, therefore showing evidence of significantly higher levels of reproductive isolation between populations compared to the pairing behaviour between male and female individuals from within each population.

However, between the SG and the N populations, there were no observations of pre-mating isolation. The above ten minute trials were then repeated, again by Weinberg *et al.* (1992), this time using the San Gabriel (SG), Newport (N) and Reish (R) populations. High levels of pre-mating isolation were observed between R and both SG and N, however there were low levels of pre-mating isolation between SG and N. It was therefore concluded that speciation occurred in the R population in the laboratory following a founder event. Weinberg *et al*. (1992) also looked at the percentage of broods that produced healthy offspring between the R, SG and N populations (a post-mating isolation experiment). A normal percentage was found to be between 75-95%. When R individuals were paired with either SG or N individuals, the percentage of healthy offspring was 0. However, when SG individuals were paired with N individuals, the percentage of healthy offspring was found to be 77%.

Due to the nature of the findings by Weinberg *et al*. (1992), suggesting that speciation of the R population had occurred in the laboratory, the findings were further discussed in the work by Rodriquez-Trelles *et al*. (1996). This work disagreed with Weinberg *et al*. (1992) and hypothesised that the two wild populations used in the study (SG and N) were not representative of the population from which the R population was originally sampled from. The study went on to investigate the genetic markers, genetic variability and divergence of the three populations. It was found that in 13/18 loci, the R population does not share any alleles with either SG or N and high genetic differences (D) were found to be as follows: SG to N = 0; R to SG = 1.75; R to N = 1.76. The genetic difference between two sibling species *N. diversicolor*, a population sampled from Germany, and *N. limnicola*, a species sampled from the U.S.A., is 1.28 (Rodriquez-Trelles *et al.*, 1996). The study by Rodriquez-Trelles *et al.* (1996) concludes that these three populations formed separate species before the R population was sampled in 1964. Further to this, work undertaken by D.H. Lunt and J.D. Hardege (unpublished) found that, based on the nuclear DNA, the C population is likely to have diverged from the other three populations approximately ten million years ago. Subsequent to this divergence, based on mitochondrial DNA, the SG population diverged from the R and N populations approximately seven million years ago. R and N have been found, based on the mitochondrial DNA, to be very similar genetically, with little divergence (D.H. Lunt and J.D. Hardege, unpublished).

One final study has looked at the pre-mating isolation between populations of *N. acuminata*. Sutton *et al.* (2005) looked again at ten minute trials between three laboratory based populations (SG, N and R) and three wild populations (Connecticut, Massachusetts and a population from Hawaii). Again, significant levels of pre-mating isolation were found between all population groups, except when individuals were paired with members of their own population. Aggression was also observed between a male and a female from different populations when placed together in the pairing experiment (Sutton *et al*., 2005). Successful courtship and pair formation depends on the recognition of cues, such as colour, posture, odour and specific sex pheromones (Weinberg *et al*., 1990). Any divergence in these traits may cause reproductive isolation and an increase in aggression (Weinberg *et al*., 1990).

Sibling species are typically difficult to distinguish based on morphological characteristics as these are often very similar (Knowlton, 1993) but examples of polychaete sibling species have been documented. *Hediste (Nereis) diversicolor*, *Hediste (Nereis) limnicola* and *Hediste (Nereis) japonica* are three morphologically similar species and form a species complex but each of these species possess different reproductive and developmental mechanisms and they have large genetic differences between them (Fong and Garthwaite, 1994). *N. japonica* reproduces via a mass spawning event (Smith, 1958), *N. limnicola* is a viviparous self-fertilising hermaphrodite (Baskin, 1970) and *N. diversicolor* females reproduce in burrows with males releasing sperm in front of the female tube (Bartels-Hardege and Zeeck, 1990).

The polychaete *Capitella capitata* has as many as six sibling species with only slight morphological differences observed between them but show large genetic differences, where only two alleles are shared by comparing any pair of the six sibling species (Grassle and Grassle, 1976). Two sibling species have also been observed in *Perinereis cultrifera* where the English Channel species and the Mediterranean species each have a unique protein binding pattern and slight variation in breeding cycles but otherwise are very similar morphologically (Scaps *et al*., 2000). Some other examples of sibling species include the rotifer *Brachionus plicatilis* (Snell and Hawkinson, 1983) and the bonnethead shark *Sphyrna tiburo* (Parsons, 1993). Sibling species have been found to react differently in response to ecological bioassays (Knowlton, 1993) so the characterisation of such species is important. The mussel *Mytilus edulis* forms part of a species complex with *M. galloprovincialis* and *M. trossulus*; *M. trossulus* is commonly mistaken for *M. edulis* (Lobel *et al*., 1990). In the study by Lobel *et al*. (1990), *M. edulis* and *M. trossulus* were collected from the same site in Canada and the body tissue analysed for twenty five different element concentrations (ppm dry weight). Due to the differences in metabolic rate between *M. edulis* and *M. trossulus*, the concentrations of the elements tested were found to vary, even though these two species were sampled from the same geographical area (Lobel *et al*., 1990).

The aim of this chapter was to understand further the interactions between male and female individuals from both within and between the four laboratory populations to ascertain the occurrence of any pre-mating reproductive isolation. Due to female death following egg shedding, mate recognition by females should be important in the species as incorrect mate recognition could lead to a wastage of gametes if eggs are shed with an ‘incorrect’ male (Snell, 1989). It was hypothesised that there would be significant pre-mating isolation between these populations when compared to pairing behaviour observed within populations. Following on from this, the final aim of this chapter was to determine if there were any pre-mating differences between the wild population Los Angeles and the laboratory reared Reish population, which has been bred in the laboratory for 44 years. These two populations were collected from the same geographical location so the LA populations may provide a better representation of the populations that R was originally sampled from. Any differences in pair formation behaviour between these two populations may further aid to decipher the differences between the populations of *N. acuminata* currently maintained in the laboratory and it is expected that these populations will have diverged and will show significant levels of pre-mating isolation. If pre-mating isolation does occur between the R and LA populations, this would add further evidence in support of the theory that the R population has speciated in the laboratory (Weinberg *et al*., 1990). Although the populations from the west coast of the USA are unlikely to encounter individuals from the east coast, it is still important to observe the behavioural interactions between the populations to attempt to understand the similarities and differences between these populations and to untangle any confusion regarding speciation between these populations. Sibling species may also react differently in response to ecological bioassays, giving varying results to standard testing conditions (Knowlton, 1993). As *N. acuminata* have been established as an indicator species for ecological testing of pollutants (Reish, 1966), it is important to know if these population groups should be treated separately to ascertain that the results obtained for such studies are valid.

## 4.2: Methodology

The populations used in this study were Reish (R), Newport (N), San Gabriel (SG), Connecticut (C) and the wild population Los Angeles (LA). Sexually mature individuals were chosen at random from population tanks containing over 100 hundred individuals and as mentioned previously, males and females were identified by their colouration by the presence of gametes in the coelom (Figure 1.3, page 25); white for males and yellow for females. Individuals were only used once in a pairing replicate. The male and female individuals were placed in a crystallising dish (70mm diameter) containing 40ml of filtered sea water (36-38‰). The size of the dish allows interaction between the two individuals but also allows for separation following an aggressive event, so neither individual is harmed by aggressive behaviour. The combinations of male and female individuals from each population can be seen in Table 4.1 below. Each pair combination was replicated 20 times (n) to provide an accurate observation of any aggressive behaviour and change in pairing behaviour over time between the four laboratory populations.

Table 4.1: Population combinations to assess pair formation behaviour.

| | | Female | | | |
|---|---|---|---|---|---|
| | | R | N | C | SG |
| Male | SG | 20 | 20 | 20 | 20 |
| | C | 20 | 20 | 20 | 20 |
| | N | 20 | 20 | 20 | 20 |
| | R | 20 | 20 | 20 | 20 |

A pin-point focal observation was made at 5 minutes, 1 hour and 24 hours to monitor aggressive interactions. If aggression was observed between a male and a female, a fifteen minute aggression score was taken using the adapted scoring system as detailed in Figure 2.1 (chapter 2, page 45). Any pairing behaviour was also noted at 5 minutes, 1 hour and at 24 hours.

To simulate natural conditions as far as possible and to maintain a constant water quality, the crystallising dish containing the male and the female was placed into an aerated jar (180mm x 100mm x 140mm) containing 2 litres of sea water (36-38‰) between the 1 hour observation and the 24 hour observation. Individuals originating from the same population were subjected to the protocol above to determine if a male and a female would demonstrate any aggressive behaviour under experimental conditions (n = 20 per population). Finally, the above protocol was repeated comparing the Los Angeles Harbour (LA) and the Reish (R) population, with reciprocal crosses between both of these populations, as detailed in Table 4.2 below (n = 20 pair combinations).

Table 4.2: Population combinations to assess pairing behaviour between LA and R populations (n = 20 pairs).

| | | Female | |
|---|---|---|---|
| | | R | LA |
| Male | R | 20 | 20 |
| | LA | 20 | 20 |

Pair formation behaviour was compared both within and between populations to ascertain levels of pre-mating isolation between the four populations. A male and a female were considered to have formed a pair when the two individuals were lying alongside one another ‘head to tail’. Comparisons were also made between the LA and R populations to ascertain any differences in pair formation behaviour between these two groups sampled from the same collection site. The total number of pairings made in this chapter equalled 360.

## 4.3: Results

### 4.3.1: Pair formation within and between the four laboratory populations.

Pairing frequencies for the three observations (5 minutes, 1 hour and 24 hours) were recorded for each of the combinations of the four laboratory population, represented in Figure 4.1 over the page. Pair formation was found to be the highest when individuals were placed with members of their original population. The Connecticut population was found to have the lowest pairing incidence when placed with individuals from other populations.

Although behavioural observations were made at three different time intervals, only the results from the 24 hour observation for each population combination were compared statistically. This was done to provide a good indication of the pairing behaviour within and between individuals from different populations without the bias of using the frequencies of individuals that may have paired at one observation point and then separated by the next. A table of the number of pairs formed after 24 hours is shown in Table 4.3 below.

Table 4.3: The number of pairs formed out of 20 replicates between males and females from the four laboratory populations (R, N, SG and C) after 24 hours.

| | | Female | | | |
|---|---|---|---|---|---|
| | | R | N | SG | C |
| Male | R | **20** | 10 | 8 | 1 |
| | N | 5 | **19** | 9 | 3 |
| | SG | 14 | 8 | **19** | 0 |
| | C | 2 | 2 | 0 | **19** |

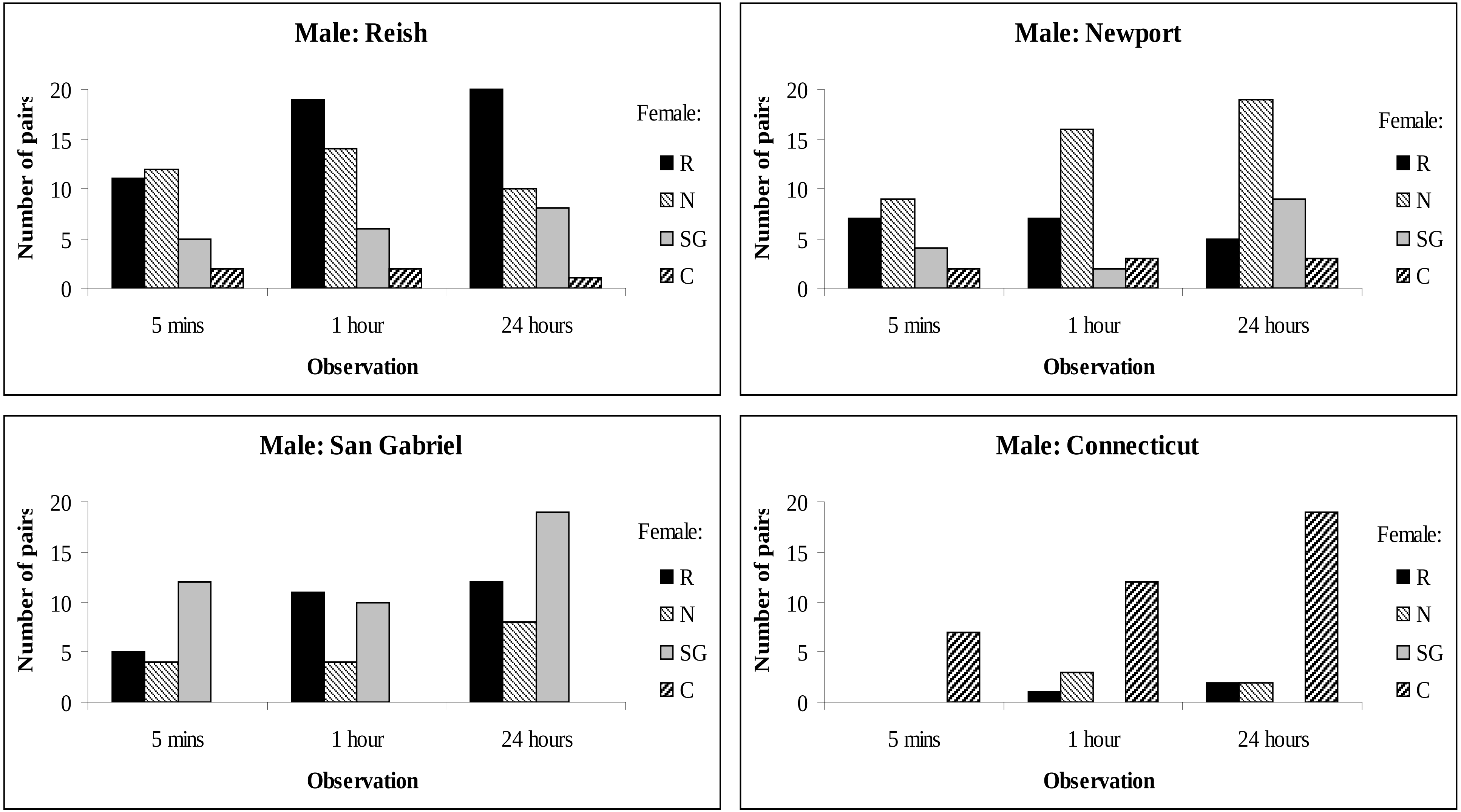


Figure 4.1: The numbers of pairs formed for each of the population combinations with observations after 5 minutes, 1 hour and 24 hours. Each combination was replicated 20 times using the Reish (R), Newport (N), San Gabriel (SG) and Connecticut (C) populations.

Pairing frequencies were converted into proportions. The arcsine transformation was then applied to each proportion (where the square root of the proportion is calculated and the inverse sin taken), shown in Table 4.4 below. The results were then analysed parametrically.

Table 4.4: Percentages, proportions and arcsine transformations for the number of pairs formed for each combination of R, N, SG and C males and females.

| Male | Female | No. of pairs | Percentage | Proportion | Arcsine transformation |
|---|---|---|---|---|---|
| R | R | 20 | 100 | 1.00 | 1.57 |
| N | N | 19 | 95 | 0.95 | 1.35 |
| SG | SG | 19 | 95 | 0.95 | 1.35 |
| C | C | 19 | 95 | 0.95 | 1.35 |
| R | N | 10 | 50 | 0.50 | 0.79 |
| R | SG | 8 | 40 | 0.40 | 0.68 |
| R | C | 1 | 5 | 0.05 | 0.23 |
| N | R | 5 | 25 | 0.25 | 0.52 |
| N | SG | 9 | 45 | 0.45 | 0.74 |
| N | C | 3 | 15 | 0.15 | 0.40 |
| SG | R | 14 | 70 | 0.70 | 0.99 |
| SG | N | 8 | 40 | 0.40 | 0.68 |
| SG | C | 0 | 0 | 0.00 | 0.00 |
| C | R | 2 | 10 | 0.10 | 0.32 |
| C | N | 2 | 10 | 0.10 | 0.32 |
| C | SG | 0 | 0 | 0.00 | 0.00 |

Pair formation frequencies observed within populations were compared to the frequencies observed between the different populations using an independent samples t-test to determine if there was a significant difference in the pairing behaviour. A significant difference was found when comparing the pairing behaviour within the populations to the pairing between the populations (t = 5.672, d.f. = 14, P = 0.000).

Further to this, to determine which population was driving the significant difference, a series of independent samples t-tests were performed, pooling the results for each combination of males and female (for example, R♂N♀ and N♂R♀ were pooled). This converts the results to 6 groups of pooled male and female combinations (with n = 40 in each case): RN, RSG, RC, NSG, NC and CSG. The percentages, proportion and the arcsine transformations were again calculated in each case and used in each t-test.

The following comparisons were therefore analysed using the t-test:
RN, RSG, RC compared to the other three groups (t = 0.888, d.f. = 4, P = 0.425).
NSG, NC, SGC compared to the other three groups (t = 0.766, d.f. = 4, P = 0.486).
SGR SGN SGC compared to the other three groups (t = 0.290, d.f. = 4, P = 0.786).
RN RC NC compared to the other three groups (t = -4.310, d.f. = 4, P = 0.013).

As these frequencies have undergone multiple testing, the Bonferroni correction was applied using the $\alpha^1$ value of 0.0125 ($\alpha = 0.05$, $k = 4$ due to the number of times each pooled population was tested) and the comparisons above were not found to be significant.

### 4.3.2: Aggression between males and females originating from different populations

Observations of aggression between a male and female were made between certain pair combinations. This was not observed when individuals were placed with a member of their own population. Aggression was found in only 16 out of 320 replicates and no aggression was observed after 24 hours.

Aggression was observed between males from the Connecticut and San Gabriel populations towards females from the Newport and Reish populations. In two instances, aggression was observed between a Newport male and a Reish female. There was also one replicate with aggression exhibited between a Reish male and a Connecticut female. The aggression level only reached level 3 in one replicate between a San Gabriel male and a Newport female (after 5 minutes and after 1 hour). After 5 minutes, 3 interactions reached level 1 aggression and 9 reached level 2. After 1 hour, 3 interactions reached level 1 and 2 interactions reached level 2 aggression (in some cases aggression was observed after 5 minutes and after 1 hour).

### 4.3.3: Pair formation between the laboratory (R) and the wild population (LA)

The results showed that pairing behaviour between the two populations R and LA were similar to the pairing observed between individuals from the same population, as demonstrated in Figure 4.2 on the next page.

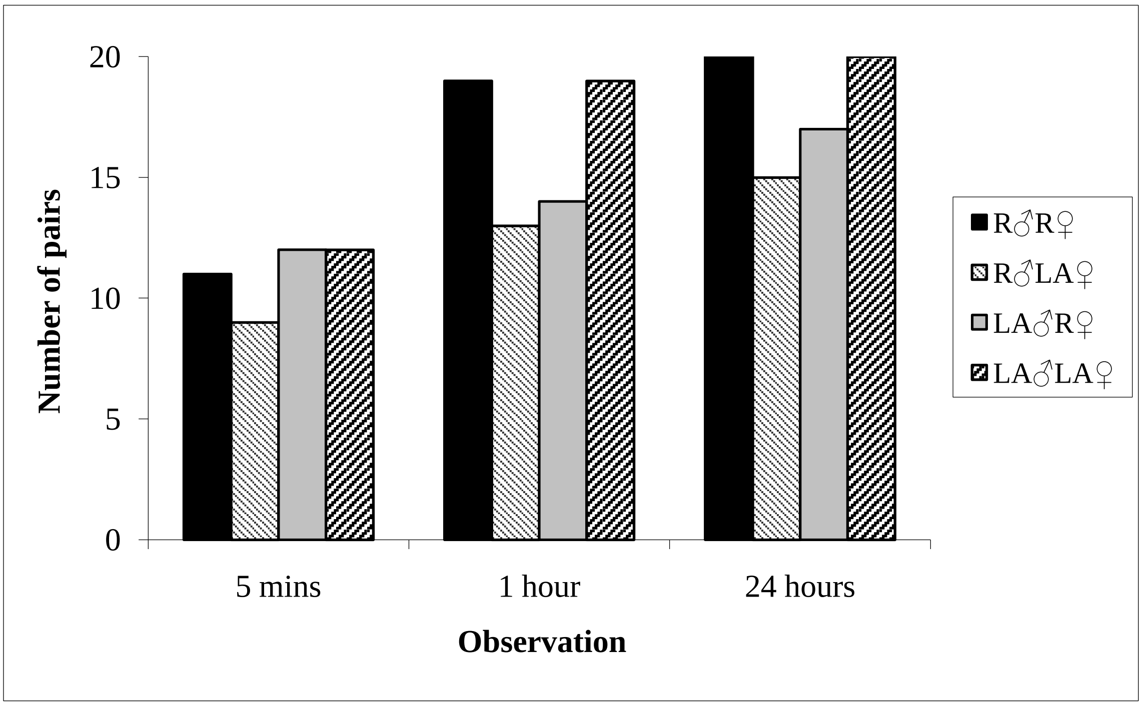


Figure 4.2: The number of pairs observed after 5 minutes, 1 hour and 24 hours between male (♂) and female (♀) individuals from the Reish (R) and Los Angeles (LA) populations out of 20 replicates for each combination.

Again, although behavioural observations were made at three different time intervals, only the results from the 24 hour observation for each population combination were compared statistically. This was done to provide a good indication of the pairing behaviour within and between individuals from different populations without the bias of using the frequencies of individuals that may have paired at one observation point and then separated by the next. The results within the R and LA populations (R♂R♀ and LA♂LA♀) were compared to the results found between males and females (R♂LA♀ and LA♂R♀). The arcsine transformation was calculated from the proportion of pairs formed and used to compare the pairing behaviour within the two populations to between them, using an independent samples t-test. Table 4.5 below shows the number of pairs formed, percentages, proportions and the arcsine transformation for each combination of males and females.

Table 4.5: Percentages, proportions and arcsine transformations for the number of pairs formed for each combination of R and LA males and females.

| Male | Female | No. of pairs | Percentage | Proportion | Arcsine transformation |
|---|---|---|---|---|---|
| R | R | 20 | 100 | 1.00 | 1.57 |
| LA | LA | 20 | 100 | 1.00 | 1.57 |
| R | LA | 15 | 75 | 0.75 | 1.05 |
| LA | R | 17 | 85 | 0.85 | 1.17 |

As this test attempted to answer a separate hypothesis regarding pair formation between the R and LA populations, no correction was made. According to Rice (1989), a correction only needs to be made when two or more tests, that cannot be pooled, are used to answer the same null hypothesis. The results of the t-test show therefore that there were significant differences in pairing behaviour when comparing pairing within populations to between them (t = 7.318, d.f. = 2, P = 0.018).

## 4.4: Discussion

Successful courtship and pair formation depends on the recognition of cues, such as colour, posture, odour and specific sex pheromones (Weinberg *et al.*, 1990). Any divergence in these traits may cause reproductive isolation (Lovern *et al.*, 1999), known as the final step before speciation (Snell, 1989). The morphological differences between populations of *N. acuminata* are minimal (Weinberg *et al.*, 1990) and populations maintained in the laboratory all share the same life history traits with monogamous mating and male parental care (Reish, 1957; Weinberg *et al.*, 1990). Therefore, both pre- and post-mating methods can be used to determine if two individuals will reproduce. In this chapter, pre-mating experiments were undertaken to determine if there were any differences in the pairing behaviour within each population compared to between them.

### 4.4.1: Pair formation within and between the four laboratory populations.

The results showed that within each of the populations R, N, SG and C, the majority of males and females had formed a pair bond after 24 hours (Figure 4.1). This was not the case when males and females from different populations were placed together. It appears that the west coast populations Reish, Newport and San Gabriel will undertake pair formation to some extent with each other (R and N = 15/40 pairs formed, R and SG = 22/40 pairs formed, N and SG = 17/40 pairs formed). Low pairing frequencies were observed when Connecticut individuals were placed with members of the other three populations, both male and female (8/120 pairs were formed). Significant differences in pairing behaviour were therefore observed when comparing the results of pairing within each population to the results of pairing between the populations. Subsequent statistical tests to determine which population was driving these differences showed that there were no significant differences when comparing each of the combinations of populations. The Bonferroni correction, however, is an overly conservative test, due to the restrictions on the critical value that is used (Rice, 1989). It is therefore likely that the pairing behaviour involving the Connecticut population was driving the significant differences observed but more replicates would need to be undertaken to ensure this. As populations are all maintained in the laboratory under identical temperature, salinity and feeding conditions, it is unlikely that these would be contributing to the differences observed in pairing behaviour between populations.

Aggression was observed between male and female individuals from different populations in 16 of the 320 replicates. Males and females may show aggression towards each other for a number of reasons. In the American lobster *Homarus americanus* for example, intersexual aggression over shelter was observed but it was found that males were more likely to win over females as males require shelter not just for predator protection but also for reproduction, providing an increased drive to win (Peeke *et al*., 1998). Male and female individuals of the cichlid fish *Cichlasoma nigrofasciatum* show aggressive behaviour that forms part of the mating ritual. The female attempts to withstand the aggressive male and in a well-matched pair, intersexual aggression is kept under control and mating can proceed (Lamprecht and Rebhan, 1997). However, intersexual aggression between a male and a female individual in polychaetes is uncommon (Weinberg *et al*., 1990). Following on from the aggression observed by males during egg incubation (Reish, 1957), similar to the male aggression exhibited by the bluefin killifish *Lucinia goodie*, where males will defend nest sites toward both male and female intruders (Fuller and Travis, 2001). In the field cricket *Gryllus integer,* males and females show aggression towards each other as part of mating (Kortek and Hedrick, 2005). Weinberg *et al*. (1990) and Sutton *et al,* (1990) observed aggression in some interactions between individuals, including males and female from the same population. This was not the case here and no aggression was observed between male and female individuals from the same population. Aggression between a male and a female may be evidence of a reproductive barrier and therefore pre-mating isolation. In this chapter, only a relatively low number of individuals were aggressive towards each other with a low level of aggression recorded, indicating that aggression is still unusual between males and females in this species. As no aggression was recorded between males and females from the same populations, it is possible that the aggressive individuals did not recognise each other as mates. It is also possible that aggressive behaviour was missed due to the experimental design; observations of behaviour were only made at three points. The populations used in these experiments were maintained in the laboratory for approximately 5 years prior to the start of experimentation. In the study by Sutton *et al*. (2005), individuals were used immediately after they were sampled from the environment. It is possible that in natural populations, males and females are aggressive towards each other but this is not observed in the laboratory after a period of acclimatisation. In the three-spined stickleback *Gasterosteus aculeatus*, habituation has been found to alter the aggression observed in individuals, with lowered aggression displayed towards nest neighbours

(Giles and Huntingford, 1985). It is possible that individuals have become habituated to the laboratory environment, altering their behaviour.

The R population maintained in the laboratory has been cultured from only six individuals (sex ratio unknown, Reish, personal communication). This population has undergone approximately 200 generations in the laboratory and is considered to be heavily inbred (Fletcher *at al.*, 2009). This may affect the results of the replicates involving the R population (either male or female) as it has been suggested that individuals avoid mating with closely related individuals to avoid inbreeding (Gerlach and Lysiak, 2006). Inbreeding depression can lower the fitness of offspring (Armbruster and Reed, 2005). In the zebrafish *Danio rerio*, kin recognition of individuals is used to avoid mating with closely related individuals (Gerlach and Lysiak, 2006). In the three-spined stickleback *Gasterosteus aculeatus*, individuals preferred mates with dissimilar Major Histocompatibility Complex (MHC) genes (Aeshlimann *et al*., 2003). The MHC is a large cluster of genes found in most vertebrates that is used by the immune system to discriminate between self and non-self (Brown and Eklund, 1994). The results showed that after 24 hours, in replicates involving the R population, 40/120 pairs were formed. In replicates involving the N and SG populations however, pairs were formed in 37/120 and 39/120 respectively, showing very similar pairing frequencies between these three populations after 24 hours. The C populations, as previously discussed, paired in only 8/120 replicates. The occurrence of inbreeding in the R population should be investigated further using choice tests between R and non-R populations to determine the breeding preferences of R population individuals.

Differences in pairing behaviour and the occurrence of aggressive displays between populations of *N. acuminata* could be due to differences at the genetic level. The Connecticut population (2n = 22) has a different chromosome number to the other populations (2n = 18) (Pesch and Pesch, 1980; Weinberg *et al*., 1990). By examining the nuclear DNA, the C population was also found to have diverged from a common ancestor earlier than the other three populations (D.H. Lunt and J.D. Hardege, unpublished). These differences in chromosome number and the divergence of the population may explain the lower incidences of pairing in replicates involving the Connecticut populations, compared to the other replicates between populations. A lack of pairing behaviour could be indicative of population divergence in this species. Nereid polychaetes have been shown to have extremely high pheromone specificity

(Zeeck *et al*., 1988) and a ‘bouquet’ of sex pheromones are detected by individuals via chemoreceptors located in the cephalic appendages and in the cirri (Boilly-Marer and Lassalle, 1980). Communication signals may be altered by the environment (Lovern *et al*., 1999). In the fruit fly *Drosophila melanogaster*, cuticular hydrocarbons are used for species recognition, courtship and mating (Billeter *et al*., 2009). Variation in the hydrocarbon profile among the nine species of the *melanogaster* subgroup causes reproductive isolation between *D. melanogaster* and these sibling species due to a lack of recognition (Billeter *et al*., 2009). As the R, N, SG and C populations were all collected from different environments which may differ in food availability, temperature, light intensity, level of predation and parasite prevalence, the signalling systems of each population may have diverged due to selection pressure for effective communication (Leal and Fleishman, 2004). Closely related species often exhibit great signal diversity and such divergence in communication signals is a factor that can promote speciation due to reproductive isolation due to a failure to communicate if such signals are important for mate recognition (Leal and Fleishman, 2004).

As successful courtship and pair formation depends on the recognition of cues, including odour and sex pheromones, any divergence in these traits may cause reproductive isolation (Weinberg *et al*., 1990). Small changes in the pheromone structure can cause major changes to occur in the effects these pheromones have on individuals and distinct populations exposed to varying habitat conditions may produce slightly different pheromones (Weinberg *et al*., 1990). The preliminary study by Brown (2005) showed that exposing individuals of the same population to different diet regimes increased the aggression observed between individuals. Male individuals fed on fish food (Tetra min) were placed in staged contests with individuals fed on low-protein processed hay with subsequent aggression levels found to be increased compared to staged contests between two individuals fed on the same diet (either fish food or processed hay) (Brown, 2005). It is therefore possible that *N. acuminata* forms a species complex comprised of different populations of individuals as individuals share the same morphology and life history traits.

Although, Weinberg *et al.* (1990) and Sutton *et al*. (2005) showed that there were significant differences in the pairing behaviour between populations after only ten minutes, in this chapter, pairing behaviour was only observed over a 24 hour time period. In these experiments, although the female used was sexually mature, it was unknown what effects the age of the female would have on the results. A female that is closer to reproducing may affect the results and pair with males at a higher frequency to protect her reproductive investment. Males and females from different populations should therefore be left for longer periods of time, using females of a precise age, with video recording equipment to monitor all interactions and behaviour between the two individuals and to determine whether aggression (if and when it occurs) is instigated by the male or the female.

The results demonstrated in Table 4.3 show that the numbers of pairs formed between populations are lower than within them, and very few pairs are formed when individuals from the Connecticut population were used in pairing experiments. The pairing behaviour demonstrated here is likely to be a more reliable indicator than aggression (Chapter 3) for determining differences between populations and may be a good starting point for comparing populations sampled from the field. As the results comparing the pairing between the populations were not significant, an increased number of replicates, using a longer time frame to investigate both pre- and post-mating behaviour are required.

### 4.4.2: Pair formation between the laboratory (R) and the wild population (LA)

Although significant differences were observed when comparing the numbers of pair formations within the R and LA populations compared to between them (Figure 4.2), there were still high levels of pairing behaviour recorded when individuals from these two populations were placed together. Within the R and LA populations, pairs were formed in 40/40 replicates. Between these two populations, pairs were formed in 32/40 interactions. No aggression was observed in any of the interactions between males and females, either within or between populations. As pairing did occur to a high frequency, and the LA population is likely to be representative of the population that R was originally sampled from, it is likely that these two populations are not isolated and speciation of the R population has not occurred in the laboratory. The two populations, although they originate from the same location, have been separated in differing environmental conditions for approximately 44 years. The R populations has been maintained under laboratory conditions for 44 years, cultured form only six individuals, whereas the LA population has been subjected to variation in temperature and light:dark cycles. Such separation of the two populations may have lead to a divergence in the behaviour in these two populations, leading to the reduced levels of pairing behaviour observed. LA Harbour is now considered to be less polluted than when the R population was sampled in 1964 due to the pollution abatement programme that was introduced in 1968 (Reish *et al*., 1980). Individuals have been shown to adapt to respond to their environment (Lahti *et al*., 2001) and the R population may have undergone certain changes to adapt to the heavily polluted harbour conditions present at the time of collection. It may be the case that R and LA individuals take longer than 24 hours to form a pair bond so this should be investigated in future experiments, leaving individuals for longer periods of time and observing to see if viable offspring are produced from pairings. Using more than 20 replicates for each combination of males and females may also help to determine if there are any differences in pair formation between these two populations.

As previously mentioned, there seems to be considerable confusion regarding the taxonomy of this species and the relatedness of sampled populations (Pettibone, 1963). There could therefore be a link between the confusion regarding the different pseudonyms used for this species and their pairing behaviour. It may be the case that populations from different locations around the world should each be called by a different species name and form part of a species complex. Individuals from different locations in this species all share similar morphology, life history traits and behavioural characteristics so other behavioural methods need to be utilised by taxonomists to determine if each population of *N. acuminata* should be described as a sibling species. It is clear that there are differences in the pairing behaviour when individuals are placed with an individual from a different population and care should be taken when attempting to compare the response of different populations to ecotoxicology testing.

### 4.4.5: Next steps

Further studies should be conducted to look into the genetic relatedness of populations and the kin recognition signals emitted by each, but also to compare laboratory raised populations of *N. acuminata* to wild populations. We do not yet know the chromosome complement of the Los Angeles population, nor the genetic relatedness of this population to the others kept in the laboratory. It would also be interesting to develop the experimental time used and leave male and female individuals for longer periods of time to determine, if individuals are left for long enough, whether they pair form after such time. Further samples of wild populations around the world should also be collected and genetic and behavioural experiments undertaken to provide a clearer picture of the diversity of populations and the occurrence of a species complex in *N. acuminata*.

Due to the experimental design used in this chapter, it is also unclear which sex is driving the significant differences observed. Further replicates with a new experimental design to determine which sex is driving isolation, is it females due to female choice, or males or both?

Post-mating isolation trials should also be undertaken to examine if offspring from reproductive events between populations results in the production of viable offspring. Although pre-mating tests give a good initial indication of population dynamics, observations of full copulation are better (Gómez and Serra, 1995). Furthermore, it would be interesting to undertake mate choice tests to see if one population is preferred over any other, especially examining the Reish population due to the fact that this is potentially an inbred population.

# Chapter Five:

## Female detection of cannibalistic males

### 5.1: Experimental reasoning and aims

Cannibalism is the consumption of conspecifics, also known as intraspecific predation (Fox, 1975). Cannibalism has been reported in a diverse range of taxa including rotifers, insects, snails, copepods, fish, birds and mammals (Fox, 1975). Population density has been found to be related to cannibalism with a positive correlation established between increases in density and the frequency of cannibalism due to an increased encounter rate (Moksnes *et al*., 1997). This has been observed in the Pacific threadfin *Polydactylus sexfilus* (Ostrowski *et al*., 1996) and the blue crab *Callinectes sapidus* (Eggleston *et al*., 2005). Due to the associated energetic gain, rates of cannibalism may also be expected to vary with changes in food availability (Polis, 1981). Although cannibalism may be costly to the cannibal, for example from increased transmission of parasites or a decrease in genetic variability due to a loss of potential mates in the population (Michimae and Wakahara, 2001), there are considered to be associated benefits with cannibalistic behaviour. It may increase individual fitness by increasing growth (Michimae and Wakahara, 2001), lowering competition for resources within a population (Fox, 1975) and/or decreasing the chances of intraspecific predation on the cannibal (Polis, 1981). Cannibalism may also act as a ‘last resort’ in a number of species when other food resources are depleted (Baras and Jobling, 2002).

Cannibalism has many forms: between non-kin individuals, between siblings, between sexual partners and between parents and offspring. Cannibalism between non-kin conspecifics, or heterocannibalism, has been observed in the wolf spider *Schizocosa ocreata* (Wagner, 1995) and the blue crabs *Callinectes sapidus* (Moksnes *et al*., 1997) and *Portunus pelagicus* (Marshall *et al*., 2005). Cannibalism between siblings has been observed in the wood frog *Rana sylvatica* where juveniles will cannibalise eggs and newly hatched individuals (Petranka and Thomas, 1995).

Intraspecific predation between sexual partners (sexual cannibalism) occurs when females consume courting males during, or immediately following copulation (Johns and Maxwell, 1997). Such cannibalism is common particularly in spiders, such as the red back spider *Latrodectus hasselti* (Andrade, 1996), but has also been observed in other invertebrate species and it may provide nutrients to the female for the production of offspring (Johns and Maxwell, 1997). Such behaviour may benefit the male consumed by increasing both the fertilisation rate and the duration of copulation. Females were also found to be more likely to reject a subsequent mate if the first mate was consumed (Andrade, 1996). The reverse (males consuming females) has not been well documented, instead males may possess weapons to harm females during copulation, which can include spiny protrusions or toxic seminal fluid, which may prevent the female re-mating or allow the male to achieve sperm precedence over previous matings (Edvardsson and Tregenza, 2005). One such example is the bruchid beetle *Callosobruchus maculatus*, where males have spines that can puncture the reproductive tract, in this case to act as a male "anchor" to prevent detachment from the female (Edvardsson and Tregenza, 2005). In the fruit fly *Drosophila melanogaster*, the male seminal fluid is toxic to the female and can cause female death (Chapman *et al*., 1995).

Cannibalism of eggs by a parent, termed filial cannibalism, may occur for a number of reasons. Aside from accidental consumption, as observed in the walleye pollock *Theraga chalcogramma* where ingestion occurs during normal gill ventilation (Schabetsberger *et al*., 1999), cannibalism may serve to clean nests of dead or diseased eggs (Kraak, 1996) but it also provides the parent with nutrients and energy (Polis, 1981). Eggs are an easily-attainable resource, rich in nutrients and are also defenceless (Acha *et al*., 2002). Parental care may be costly in terms of reduced foraging opportunities (Lindström, 1998) so filial cannibalism may also function to offset the cost of such care (Okuda and Yanagisawa, 1996). In many fish species, eggs are carried in the oral cavity, known as mouthbrooding (Okuda and Yanagisawa, 1996). This forces the parent to fast during egg care and may lead to a deterioration of physical condition (Okuda *et al*., 1997). Consuming eggs may not fully compensate for the cost of care (Kraak, 1996) but has been found to have the potential to increase future reproductive success by survivorship of the parent (Lindström and Sargent, 1997). Such cannibalism is known as partial clutch cannibalism but during egg care, whole clutch cannibalism may also occur (Payne *et al*., 2002). Whole clutch cannibalism has been interpreted as a decision by the parent to terminate care of eggs, with energy therefore redirected towards attracting new mates or defending a nest site for longer (Peterson, 1990). In the cardinal fish *Apognon doederleini*, Okuda and Yanagisawa (1996) found that males were more likely to cannibalise eggs if they were expecting to re-mate quickly. When mate availability is high, males can easily obtain matings from additional females (Kondoh and Okuda, 2002). Whole clutch cannibalism may also occur when the brood is smaller than normal as the reproductive value of the clutch would not outweigh the costs associated with care of that brood (Petersen and Marchetti, 1989).

Filial cannibalism is more prevalent in species with male parental care (Okuda *et al.*, 1997) due to the large gametic investment made by the female compared to the smaller parental investment made by the male (FitzGerald, 1992). Some examples of egg cannibalism in species with male parental care include the assassin bug *Rhinocoris tristis* (Thomas and Manica, 2003), the cardinal fish *Apognon notatus* (Okuda, 2000) and *Apognon lineatus* (Kume *et al.*, 2000) and the beugregory damselfish *Stegastes leucostictus* (Payne *et al.*, 2002). To counteract losses from predation and cannibalism, females have been observed ‘copying’ the mate choice of previous females by laying eggs in nests already containing egg masses (Rohwer, 1978). Such behaviour has been observed in teleost fish and increases the chances of a female selecting a good quality male. Such copying also increases the level of care to the brood due to its larger size and dilutes the predation risk (Kraak, 1996). Females have also been observed using test eggs, small clutches containing only a few eggs, to test the parental abilities of males (Kraak and van den Berghe, 1992; Manica, 2010). Kraak and van den Berghe (1996) reported that in the blenny *Aidablennius sphynx*, females lay small clutches of one to ten eggs in nest sites defended by males (‘normal’ clutch sizes laid contain > 100 eggs). Males were significantly less likely to receive additional eggs if the small clutches were not present after one day, compared to those received when the small clutches remained (Kraak and van den Berghe, 1992). Females therefore test the quality of male care by laying such clutches and males should therefore be prepared to care for small numbers of eggs in order to increase their chances of receiving additional matings (Kraak and van den Berghe, 1992). The laying of such test eggs has also been reported in the scissortail sergeant *Abudefduf sexasciatus* where eggs are also used to monitor male parental care quality (Manica, 2010). Although detailed in the examples above, few studies have gone into detail regarding sexual selection by females for or against cannibalistic males.

In the experimental species *N. acuminata*, filial cannibalism has been observed with males caring for offspring (Oshida *et al.*, 1981) but why and how such behaviour in this species occurs has not been fully examined. Personal observations of cannibalism of eggs by the male have indeed been made during egg care with both experienced and inexperienced males, with the male consuming the whole clutch in every case. Female

*N. acuminata* make a larger gametic investment than males due to female death following reproduction (Starczak, 1984) whereas males make a larger parental care investment than females (Reish, 1957). Females of this species have been observed to select males based on male experience of parental care, preferring males that have successfully reared a brood to the larval stage (Fletcher *et al.*, 2009). If female choice occurs, females should select for good quality males (Hoelzer, 1989). Filial cannibalism may be a way for males to increase their physical condition prior to undertaking future parental care (Klug and St. Mary, 2005) and this may serve as an advantage to *N. acuminata* females looking for a male able to undertake sole parental care once the female has laid her eggs and died. Males in good physical condition are less likely to need to cannibalise eggs during egg care (Manica, 2010). Females should minimise the chances of the reproductive investment being lost due to the eggs laid becoming food (Petersen, 1990). The occurrence of male filial cannibalism can therefore exert a selective influence on female reproductive tactics (Petersen, 1990).

As males of the experimental species have been found to cannibalise eggs, the first aim of this chapter was to determine if there was an effect of the presence of a cannibalistic male on aggression when such males are placed with either an inexperienced male (one that has not been allowed to reproduce) or an experienced male (one that has been allowed to complete one brood cycle). This was then compared to the aggression observed within and between experienced and inexperienced males. It was expected that as males consuming eggs should be in good physical condition, a reduction in the occurrence of escalated aggression would be observed. The presence of a male potentially in better condition would alter the Resource Holding Potential (RHP) of that male, causing there to be asymmetry between the opponents. Such asymmetries due to differences in the RHP of opponents can lead to the quick resolution of fights (Maynard Smith, 1974). The second aim of this chapter was to examine if a female can detect that a male has cannibalised an egg mass under his care and if so, whether this influences female choice of male, with females preferring to mate with either an inexperienced or an experienced male over a cannibalistic male. As sole male parental care occurs in this species (Reish, 1957) and females make a larger gametic investment than males (Starczak, 1984), the female should select a male in good physical condition, capable of rearing her brood (Hoelzer, 1989). Female mate choice trials were therefore observed to determine if a female could detect that a male has cannibalised an egg mass under his care and if so, whether the female would still form a monogamous pair bond with such a male.  As females should select a male based on his ability to care for her brood to avoid her reproductive investment being wasted (Hoelzer, 1989), and as males that consume eggs may be in better physical condition (Klug and St. Mary, 2005) and less likely to cannibalise eggs during future egg care (Manica, 2010), it was expected that females would not avoid pairing with a male that had previously cannibalised an egg mass.

## 5.2: Methodology

Males were separated from the Newport (N) population at the larval stage and isolated to ensure that they did not undertake reproduction. These were kept in isolation and noted as ‘inexperienced’ males. Once a male had become sexually mature, he was placed in a crystallising dish (70mm diameter) containing 50ml of sea water (36-38‰) with a sexually mature female (selected at random from the N population). The dish was then placed in an aerated jar (180mm x 100mm x 140mm) containing approximately 2 litres of sea water (36-38‰) to maintain a constant water quality. The male and female were allowed to form a pair and reproduce with observations made every day to detect the presence of eggs in the parental burrow. If males were observed to have ingested the egg mass, confirmed visually to the naked eye by the presence of eggs in the gut contents, they were subsequently noted as ‘cannibalistic’. If the male completed egg care and emergent juveniles were observed around the parental tube, the male was noted as ‘experienced’.

Two distinct sets of replicates were performed using males that had cannibalised an egg mass. A cannibalistic male was placed in a crystallising dish (70mm diameter) with 50ml of sea water (36-38‰) with a sexually mature female and another male (either inexperienced or experienced). The three individuals were then observed over a fifteen minute time period and an aggression score of the interaction between the two males taken (as detailed in Figure 2.1, page 45). The highest level of aggression was noted for each fight, as follows:

Level 0: no aggression between the two individuals.
Level 1: individuals avoid contact with each other, palpi may be slightly flared.
Level 2: a fighting position is assumed by both individuals with the palpi flared and the jaws visible.
Level 3: severe aggression is observed with biting of the opponent.

Female choice of male was also observed, noted when the female formed a pair bond with a male, lying ‘head-to-tail’ with the other individual. The trials were repeated, with inexperienced (n = 20) and experienced (n = 10) males placed with the cannibalistic male and the female.

The highest levels of aggression reached were also examined in a further 3 trials: between two inexperienced males (n = 20); between two experienced males (n = 20);

and between one inexperienced and one experienced male (n = 20). The above protocol was followed with individuals observed over a fifteen minute time period, with a female present, to determine if interactions involving cannibalistic males exhibited different aggression levels. The total number of replicates equalled 90.

## 5.3: Results

The percentage number of fights reaching each aggression level for cannibals versus inexperienced males (n = 20 fights) and cannibals versus experienced males (n = 20 fights) is shown in Figure 5.1 below, compared to the highest aggression levels recorded between two inexperienced males, two experienced males and between an inexperienced and an experienced male. The highest aggression levels reached in each trial were found to vary depending on the two males placed together. There were generally low incidences of level 3 aggression exhibited, except between two experienced individuals (20% of replicates) compared to that exhibited in the trials involving cannibalistic males (5% with inexperienced males and 0% with experienced males). With aggression between two inexperienced males, there were low incidences of level 2 and 3 aggression (5% and 10% respectively) and therefore more interactions exhibiting level 1 aggression (45%) or no aggression at all (40%). With aggression between inexperienced and experienced males, no fights reached level 2 or level 3, with most fights reaching level 1 (65%).

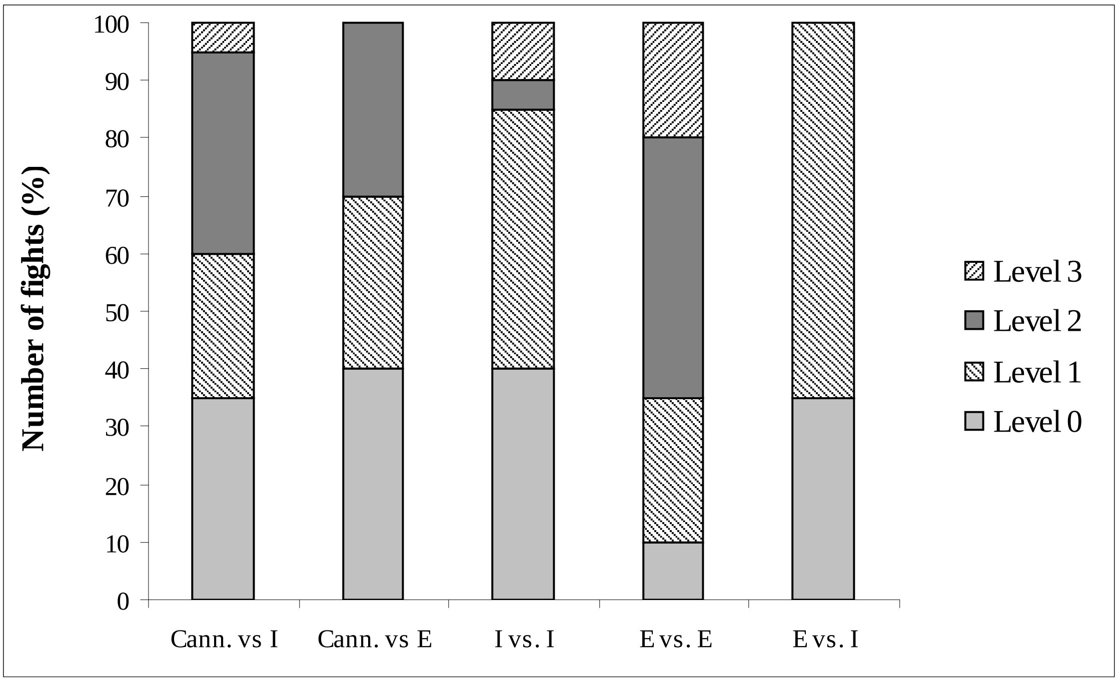


Figure 5.1: The percentage of number of fights reaching each level of aggression (0, 1, 2 or 3) when males from different reproductive states were placed together: inexperienced (I), experienced (E) and cannibalistic (Cann.) males.

The aggression levels exhibited in all five trials (Cann. vs. I, Cann. Vs. E, I vs. I, E vs. E and E vs. I) were compared using a Kruskal-Wallis test and significant differences in the highest aggression level reached were found between the groups ($\chi^2/_2$ = 16.466, d.f.

= 4, P = 0.002). Using non-parametric comparisons with unequal sample sizes outlined in Zar (1996), the mean ranks of aggression between males for each group were compared. The standard error was taken as 7.830 (for $n_1$ and $n_2 = 20$) or 9.589 (for $n_1 = 20$ and $n_2 = 10$) with a critical value of $Q_{0.05,5} = 2.807$. Significant differences in aggression levels were found between the E x E group and the E x I groups (Q = 3.675) and also between the E x E groups and the I x I group (Q = 3.254). There were no significant differences between any of the other groups (Q ranging between 0.421-2.289).

The percentage of males selected by the female for each of the 2 trials involving cannibals is shown in Figure 5.2 below. When females were given a choice between an inexperienced and a cannibalistic male, the female selected the cannibalistic male in 50% of the replicates. When females were given a choice between experienced and cannibalistic males, the female selected the cannibalistic male in 40% of the replicates. The choice of male made by the female for each trial was compared using the Chi-squared test. There was no significant difference in female choice between inexperienced males and cannibalistic males ($\chi^2/_2 = 0.000$, d.f. = 1, P = 1.000). There was also no significant difference in female choice between experienced males and cannibalistic males ($\chi^2/_2 = 0.400$, d.f. = 1, P = 0.527). It was therefore found that there was no preference for one male over another in either of these trials.

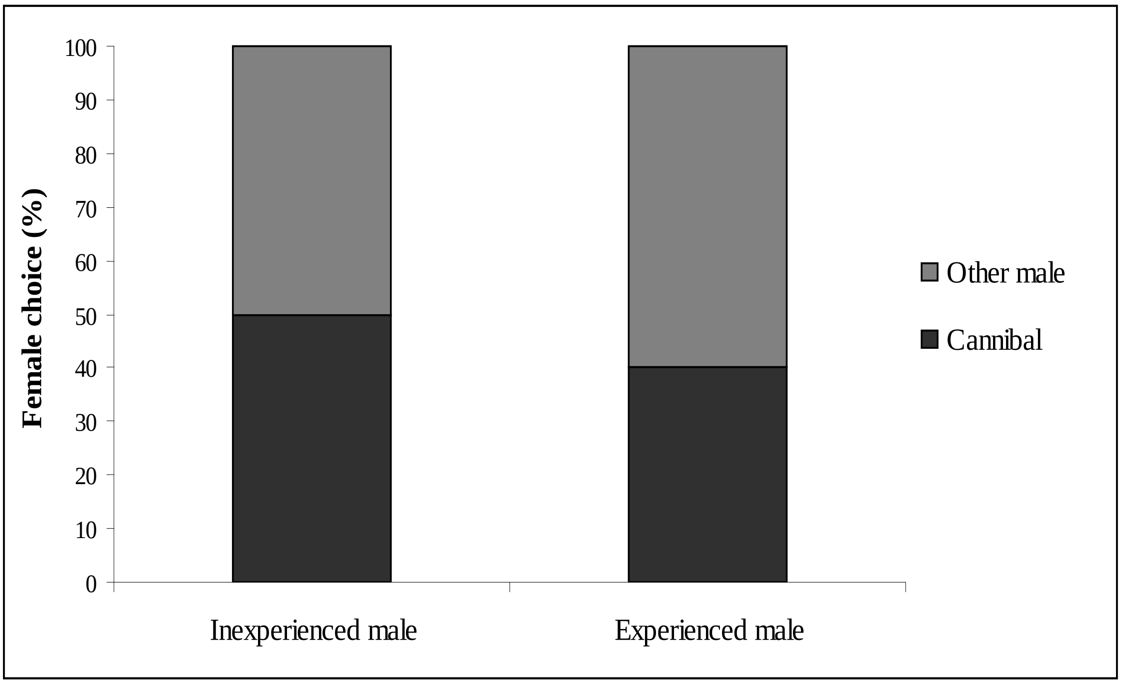


Figure 5.2: The percentage of males selected in each trial for inexperienced vs. cannibalistic males and for experienced vs. cannibalistic males.

Aggression was also observed between a cannibalistic male and a female in some of the replicates, which was not expected. The aggression observed between a female and a cannibalistic male was scored the same as that between two males, with the highest aggression level reached noted over the course of the 15 minutes observation. In 4 out of 20 of the inexperienced versus experienced trials, an aggressive response was noted between the female and the cannibalistic male. The highest aggression reached was as follows: level 1 (1 case), level 2 (2 cases) and level 3 (1 case). In all of these trials, the female selected to form a pair with the inexperienced male. Aggression between a female and a cannibalistic male was only seen in 1 trial out of 10 experienced versus cannibalistic males, reaching level 2 aggression. The female selected the experienced male in this replicate.

## 5.4: Discussion

Significant differences in aggression levels between males from different reproductive states were found (Figure 5.1). However, post-hoc testing showed that significant differences were only observed when comparing the aggression with experienced males (E x E) to that with an experienced male and an inexperienced male (E x I) and to that with two inexperienced males (I x I). The aggression between two experienced males showed more fights reaching level 2 and 3. This is in contrast to the fights between I x I and E x I in which both groups had aggression levels reaching only level 1 and most showing no aggression (level 0).

Game theory predicts that individuals should compare their Resource Holding Potential (RHP) with that of their opponent before escalating aggression both before and during a fight (Sneddon *et al.*, 1997). RHP may be influenced by morphology, body size physiology, age, sex (Beacham and Newman, 1997), prior residency, resource value, energy reserves, physical condition and previous aggressive encounters (Morrell *et al.*, 2005). Relative body size, including weapon size can also influence RHP (Sneddon *et al.*, 1997). Contests are predicted to be resolved more quickly the more the strengths and weaknesses of the opponents differ (Gammell and Hardy, 2003). Aggression displays between individuals with similar potentials however, are likely to be escalated and last longer (Sneddon *et al.*, 1997). In the fiddler crab *Uca mjoebergi* for example, individuals that were more closely size-matched fought for longer than pairs where there was a large size discrepancy (Morrell *et al.*, 2005). In the shore crab *Carcinus maenas*, the chelae (weapons used in aggression) grow in relation to body size (carapace width) and were found to be a more reliable indicator of the outcome of fights in pair-wise contests (Sneddon *et al.*, 1997). In *N. acuminata*, the jaw would be considered weapons as these are used in aggressive interactions and therefore the ability to win fights may not be related fully to body size but instead related to weapon size. In this experiment, with aggression between two experienced males, RHPs are likely to be similar due to similarities in age, size, reproductive state and potential weapon size. Interactions between closely matched individuals typically escalate over time (Stocker and Huber, 2001). This is also supported by the lowered aggression observed between experienced and inexperienced males. If one individual has a higher RHP than another, fights are predicted to be shorter in order for the loser to conserve energy and reduce the costs associated with fighting (Caldwell, 1989). In the case of aggression between two inexperienced males in this experiment, fights were found to exhibit very low levels of aggression. Although RHP values are predicted to be the same between these individuals, escalated fights may be avoided due to their reproductive state; inexperienced males may be naturally less aggressive. Aggression between *N. acuminata* individuals is therefore very complex and although individuals were size-matched during replicates, a host of other variables may not have been taken into account when observing aggressive interactions.

The results (Figure 5.1) showed that there were no significant differences in the aggression exhibited between cannibalistic males and either inexperienced or experienced males. The same amount of aggression was expressed between cannibals and males of both reproductive states. This indicates that the presence of a cannibalistic male does not change the aggressive behaviour exhibited, either because a cannibalistic male is not recognised as such, or the resources competed for are not altered by one male being cannibalistic.

The results also show that there were no significant preferences exhibited by the female either for or against cannibalistic males (Figure 5.2), regardless of the reproductive state of the other male in the trial. When females were placed with a cannibalistic male and an inexperienced male, the female choose the cannibal in 50% of the replicates. When females were placed with a cannibalistic male and an experienced male, the female choose the cannibal in 40% of the replicates. Although sexual selection by females has been discussed in terms of male mating success and the benefits of cannibalism to male physical condition, female preferences for or against cannibalistic males have been sparsely discussed in the literature. Several theories related to sexual selection are suggested to attempt to explain the behaviour of the females in this chapter.

Females should want to select the best possible male to care for her eggs as the female dies following egg release, leaving parental care solely down to the male (Reish, 1957). If the female selects a male that isn’t able to care for her brood, the reproductive effort of the whole of her lifetime is wasted. Females may not be able to detect that males are cannibalistic males; perceiving a male that has eaten eggs as inexperienced. This would explain why cannibalistic males and inexperienced males were chosen equally. However, this does not explain the results observed when females were choosing between cannibalistic males and experienced males. If females cannot detect that a male has cannibalised his eggs, then experienced males would be preferred over cannibalistic males, as cannibalistic males have not produced a larval brood. Although males have consumed their egg mass, it could be the case however that such males have undertaken a partial form of care. Fletcher *et al*. (2009) found that experienced males were preferred over inexperienced males and that this advantage was conferred to the female chemically via a 'scent of experience'. Although full care has not been completed, *N. acuminata* cannibalistic males may convey this 'scent of experience' to females. This chemical signal may also be related to physical condition, with males in good physical condition, and with experience of producing a viable brood, being preferred over other males. Cannibalistic males may give an indication to the choosing female that he was attractive to a previous female and this may also be involved in the 'scent of experience'; a female is selecting a male that a previous female has found attractive and that females copy the mate choice of previous females, as in the three-spined stickleback *Gasterosteus* aculeatus, where females lay eggs in nests already containing egg masses (Jamieson and Colgan, 1989). It is unknown in *N. acuminata* whether males have to complete a full brood cycle, producing viable offspring, to become experienced and become preferred by females. Cannibalistic males may be signalling to the female that they are capable of undertaking parental care and are in good physical condition. This would explain why cannibalistic males were selected in 40% of cases against experienced males, if both are equally adept at caring for the female's brood. In this instance, if cannibalistic males were selected by females either by caring for a brood partially, or as a form of copying, it would be expected that cannibalistic males would be preferred over inexperienced males. This was not observed here as there was no significant difference in female selection when given a choice between an inexperienced male and a cannibalistic male.

The female may be 'confused' by cannibalistic males. After eggs are eaten by the male, they are visible in the gut. It may be that the presence of eggs in the gut, or the colour or smell, indicates to the female that there is another female present which is in fact the cannibalistic male. This is supported by the aggression exhibited between females and cannibalistic males in some of the trials. Aggression between males and females has been observed when males are undertaking egg care (Reish, 1957) and as discussed in Chapter 4 between males and females from different populations. However, aggression between the two males was also observed in all but one of these trials so the presence of eggs in the gut has not 'confused' the other male present. Aggression between a male and a female was only seen in 5 trials out of 30 replicates in total. If females were confused by the presence of eggs in the coelomic tract, more instances of aggression between males and females would be expected. It is possible that these occurrences of aggression are an anomaly and on occasion, male and female *N. acuminata* will fight with each other over space for example. Such aggression between males and females has previously been observed in the American lobster *Homarus americanus*, where males and female will fight over access to shelters (Peeke *et al*., 1998).

It is possible that females can detect the presence of a male that has cannibalised his brood. Filial cannibalism can occur for a number of reasons and not all of these should be considered selfish on the part of the individual consuming the eggs. Although eggs are a good source of food and can increase growth and fitness (Polis, 1981; Michimae and Wakahara, 2001), filial cannibalism may also be undertaken to clean the nest of dead and diseased eggs to prevent the spread of infection (Kraak, 1996). Such cannibalism may also occur if the costs of care outweigh the benefits received, as with small clutches with low viability (Petersen and Marchetti, 1989) or may occur accidentally (Schabetsberger *et al*., 1999). Filial cannibalism has been thought to increase the future reproductive success of the parent by survivorship (Lindström and Sargent, 1997). Mating with a cannibalistic male may be beneficial to the female. It may be that cannibalistic males are in better physical condition due to the ingestion of eggs and they may be potentially better at looking after a future brood. If eggs were in poor condition or were diseased, filial cannibalism may indicate to a female that the male has good parenting skills if eggs in such condition were consumed (Kraak, 1996). Males in good physical condition may be less likely to consume eggs when undertaking care of future broods (Okuda *et al*., 1997) and filial cannibalism may be a way for males to increase their physical condition prior to undertaking parental care (Manica, 2010). The occurrence of cannibalism in *N. acuminata* may not be related to female choice and the female may be selecting between two males, choosing the male in better physical condition. The occurrence of continued filial cannibalism in this species has not been reported; it may be the case that males that cannibalised eggs go on to produce viable offspring in subsequent matings. Cannibalistic males may form a ‘middle ground’ between inexperienced and experienced males; males that have cannibalised their egg mass may have undertaken some form of care but have not produced a viable brood.

It is clear that female choice in this species is altered in the presence of cannibalistic males but possible explanations for the behaviour observed are not supported clearly by the results, either in trial involving experienced or inexperienced males. More replicates should therefore be carried out using cannibalistic males. It could be the case that performing the experiment with more replicates may show that females prefer experienced males over cannibalistic males. Therefore, further experimental studies need to be undertaken to understand the processes involved.

### 5.4.1: Next steps

Further replicates need to be undertaken between cannibalistic males and experienced males to determine if, with more replicates, there is a female preference for experienced over cannibalistic males. It is also unclear at what stage males consume their eggs and what affect this has on their subsequent mating success and why inexperienced males would consume their egg brood unless the costs of care were too great. The physical condition of the males, nor the reasons why males cannibalised their eggs were not known prior to this experiment. This needs to be investigated as differences between males looking for additional food and males cleaning the nest of dead and diseased eggs may cloud the results. It may also be necessary to conduct trials using all males in the three different reproductive states: inexperienced, cannibalistic and experienced. This may help understand what drives female choice in *N. acuminata*.

Females may lay clutches of different sizes due to variation in body size and growth rates of females. In future experiments, an attempt to control this should be investigated in the future. If males can care for a brood for a short period of time and become ‘experienced’, egg cannibalism may occur more frequently to gain both nutritional benefits and increased reproductive success. Such behaviour needs to be examined in this species involving choice trials with females correlated with the time spent caring for eggs. Future experiments should also examine if there is a repeated occurrence of filial cannibalism in *N. acuminata* individuals: are cannibals always cannibals? As aggression has been exhibited between females and cannibalistic males, further experiments need to be carried out to determine the cause of this; whether males that have ingested eggs are perceived as female, whether the ‘scent of experience’ is involved and how males and females detect one another. Fundamentally, it is still unknown how individuals can recognise when they are in the presence of a male or a female.

# Chapter Six:

# The effect of the scent of experience on aggression and female mate choice

### 6.1: Experimental reasoning and aims

Females have been shown to select mates based on dominance ranking in many species (Searcy, 1982). It may be that dominant males possess a greater ability to provide breeding resources such as territory and female protection (Moore *et al.*, 2003) and dominance may also be a good indication of reproductive success in males (Searcy, 1982). Dominant males are likely to be larger in size and this may indicate that they are superior at fighting for access to mates (Bisazza *et al.*, 1989) or that they are better able to provide protection against predators (Takahashi and Kohda, 2001). Females have also been found to "eavesdrop" on aggressive contests between males to gain information regarding mate quality, as observed in the Siamese fighting fish *Betta splendens* (Doutrelant and McGregor, 2000). This ensures that females mate with high-quality males (Forsgren, 1997) as success in contests is indicative of good male condition (López *et al.*, 2002).

However, in some species, although male to male contests and the formation of dominance hierarchies have been observed, females do not always select for the dominant individual (Moore *et al.*, 2003). In the three-spined stickleback *Gasterosteus aculeatus*, females show no preference for dominant males over subordinate males, selecting males that were less aggressive (Ward and FitzGerald, 1987). In species with male parental care, the ability to raise offspring may be the deciding factor in mate choice, with good fathers preferred (Wong, 2004). Hatching success must be heavily reliant on male care for females to select mates based on their parental care ability and therefore, the selected trait conferring the signal to the female must be reliable (Hoelzer, 1989). Dominance may not provide such a reliable indication of parental care ability (López *et al.*, 2002) and dominant males do not always make better fathers (Wong, 2004). Hatching success in the sand goby *Pomatoschistus minutus* has been shown to be dependent on sole paternal care and males with nests containing eggs are preferred over those with empty nests (Forsgren *et al.*, 1996). Added to this, dominant males were not found to be better at egg care and females gained no direct benefits from mating with such males (Forsgren, 1997).

The life cycle of *N. acuminata* has been extensively studied (Reish, 1957). It is known that females die following egg release, whilst males undertake sole parental care of eggs and can then reproduce again (Reish, 1957). Once males have reared a brood, they are referred to as 'experienced' (and those that have not 'inexperienced'). It has been shown in *N. acuminata* that females prefer ‘experienced’ males over ‘inexperienced’ males (Fletcher *et al*., 2009), even though there are high levels of reproductive investment made by both males and females (Starczak, 1984). Female reproduction costs due to female death following egg dehiscence are likely to be higher than costs incurred by males. Males are also able to reproduce again following completion of egg care (Reish, 1957), meaning males have a lower investment than females (Starczak, 1984). There is also evidence that choice is based on chemical cues received by the female (Starczak, 1984; Fletcher, 2004; Storey, 2006). Although these findings add support to the evidence that female choice occurs in this species, it is still not entirely clear at which point the male becomes preferred by a female, either by a morphological, behavioural or hormonal change in the male, and if such a preference is signalled to the female chemically.

Conditioned water bioassays have been used previously to determine if water-borne chemicals are used for communication both between and within species. Such experiments use surrounding water taken from certain individuals that can then be used in choice trials to determine the effect (if any) of chemical signals that may be present in the water sample. In the zebrafish *Danio rerio*, Gerlach and Lysiak (2006) used conditioned water from four different kin groups in a choice chamber to determine if there was a difference in preference between males and females. Females were found to prefer non-related males whereas males were indiscriminate of kin group. In another study, the sea urchin *Strongylocentrotus droebachiensis* moved away from conditioned water from crab and lobster predator sources compared to the control with no stimulus. Urchins were also shown to respond to stimuli from damaged urchins, moving away from the odour source, likely to be an anti-predator response (Mann *et al.*, 1984). Odour choice tests have also been examined in the brown trout *Salmo trutta*, where individuals preferred the odour of same-sibling groups to non-siblings. Individuals of this species how been found to prefer to shoal with same-sibling groups (Ojanguren and Braña, 1999). Studies on the velvet swimming crab *Necora puber* have shown that conditioned water originating from sexually mature females influenced aggressive interactions between two competing males, increasing aggression between them as there was a perceived presence of a female and therefore a perceived resource to induce further aggressive bouts (Smith *et al*., 1994). Also with crustaceans, in the blue crab *Callinectes sapidus*, females aggregate to release egg masses and ovigerous females have been found to respond positively to odour plumes of water conditioned with such masses (Tankersley *et al*., 2002).

Conditioned water has also been used to examine the behavioural responses in *N. acuminata*. Brown (2005) found that aggression was lowered between males originating from different populations when exposed to conditioned water from the opposing population. It was also found that female choice changed in *N. acuminata* when males were placed in conditioned water originating from males undertaking parental care of an egg mass. Previous losers of female choice were subsequently chosen by the female after they were placed in conditioned water from parental males (Fletcher *et al*., 2009).

The aims of this chapter were, first, to observe if there was any change in aggression following the masking of a male with conditioned water from various sources. Individuals use assessment strategies to determine the RHP of their opponent and the likelihood of winning a fight (Maynard Smith, 1974). Placing males in conditioned water from females, juveniles and inexperienced males was not expected to alter the aggressive behaviour observed between individuals, as the RHP of the masked opponent (an inexperienced male) should not be altered by the conditioned water. However, conditioned water from males caring for eggs and both new and old experienced males was expected to alter the aggression observed between two opponents as the RHP of the masked male would have changed due to the 'presence' of a male undertaking egg care or an experienced male. Second, to examine if female preference was artificially altered following the use of such conditioned water and if a water-borne chemical cue is involved in female choice and reproduction in *N. acuminata*. Experienced males convey chemically to females that they have previously undertaken egg care, as previously shown with conditioned water from males caring for eggs (Fletcher *et al*., 2009) but it is unknown if the same effect will be observed with conditioned water from other sources. Individuals in different stages of the life cycle of *N. acuminata* were used to condition water and these include juveniles, sexually mature females, inexperienced males, males undertaking egg care, new experienced males and older experienced males. Conditioned water from older males was also used to observe if there was an effect on female choice due to the age of the male. As females have been shown to prefer experienced males over inexperienced males, it was expected that males placed in conditioned water from new and old experienced males would alter subsequent female choice, as would the conditioned water from males caring for eggs. It was expected that the conditioned water from females, juveniles and inexperienced males would not alter subsequent female choice as these should not alter the initial choice of mate made by the female.

## 6.2: Methodology

All individuals used in this chapter originated from the Newport (N) population and experiments were undertaken in the laboratory at CSULB. Two inexperienced, size-matched males and a sexually mature female were placed in a crystallising dish (55mm diameter) with 50ml of filtered sea water (36-38‰) and observed for fifteen minutes. Males were identified as 1 and 2 using the natural variations in the faecal matter in the gut of each individual to allow the observer to distinguish between them. An aggression score for the two males was recorded over a period of fifteen minutes using visual observations to ascertain the highest level of aggression between the two individuals. As detailed in earlier chapters (2, 3 and 5) and shown in Figure 2.1 (page 45), the following scoring system was used:

Level 0: no aggression between the two individuals.
Level 1: individuals avoid contact with each other, palpi may be slightly flared.
Level 2: a fighting position is assumed by both individuals with the palpi flared and the jaws visible.
Level 3: severe aggression is observed with biting of the opponent.

Female choice for male 1 or male 2 was also noted when the female formed a pair bond with a male, where the two individuals lie alongside each other, head to tail (as shown in Figure 3, page 49). The three individuals were then separated and placed in 70mm diameter crystallising dishes containing the following:

| | |
|---|---|
| Female: | 50ml filtered sea water |
| Winner of female choice: | 50ml filtered sea water |
| Loser of female choice: | 50ml conditioned sea water |

Following separation, the fifteen minute aggression test was repeated and female choice was noted to observe if there were any changes in female choice and/or male aggression behaviour following the use of conditioned water. Each treatment (A-G, listed below) was replicated twenty times. The total number of trials undertaken in this chapter equalled 140.

Conditioned water was produced by placing individuals from each of the treatment groups in 50ml of pasteurised sea water (salinity 36-38‰) in a crystallising dish (70mm diameter) for 15 minutes. Pasteurised water was produced by heating sea water on a hot plate with a mixer at 80°C for 2 hours, allowing the water to cool to 18°C before use (normal tank water temperature). Pasteurised water was used to remove any impurities or live contaminants that could potentially influence the results of the experiment. The experimental treatment groups used to obtain conditioned water were as follows:

**A**: Juveniles: 10 individuals in 50 ml pasteurised sea water (10 individuals were used for this treatment as juveniles are approximately 10 times smaller than adult worms).

**B**: Sexually mature female water: 1 individual in 50ml pasteurised sea water.

**C**: Inexperienced males: 1 individual in 50ml pasteurised sea water.

**D**: New experienced males: 1 individual in 50 ml pasteurised sea water (males were noted as experienced when emergent juveniles were observed).

**E**: Old experienced males: 1 individual in 50 ml pasteurised sea water (males were isolated from the population following completion of egg care and used in trials after at least two weeks).

**F**: Male caring for eggs: 50ml of sea water was taken from a crystallising dish containing the parental burrow with eggs.

**G**: Control: pasteurised sea water was also used as a control to determine if any changes observed were due to the use of the conditioned water treatments rather than due to the separation of the three individuals or due to any variable that may have been unaccounted for.

New experienced males were obtained by removing males from the parental tube once hatched larvae had been observed outside of the burrow. A portion of the males that produced a successful brood were kept in isolation for 2 weeks and then noted as old experienced males. Conditioned water was made when necessary for experimental replicates and discarded after each trial. Inexperienced males were separated as juveniles and kept in isolation from all other individuals to ensure they did not reproduce. Inexperienced males were placed with females and allowed to reproduce to produce experienced males and also kept in isolation following the completion of egg care.

## 6.3: Results

As shown in Figure 6.1 below, the aggression levels between males varied between the pre- and post-treatment observations (A-G). Level 0 and level 1 aggression were generally observed more frequently than both level 2 and level 3, with level 3 aggression generally very rare for each treatment, for both pre- and post-treatment observations following placement of the loser male in conditioned water.

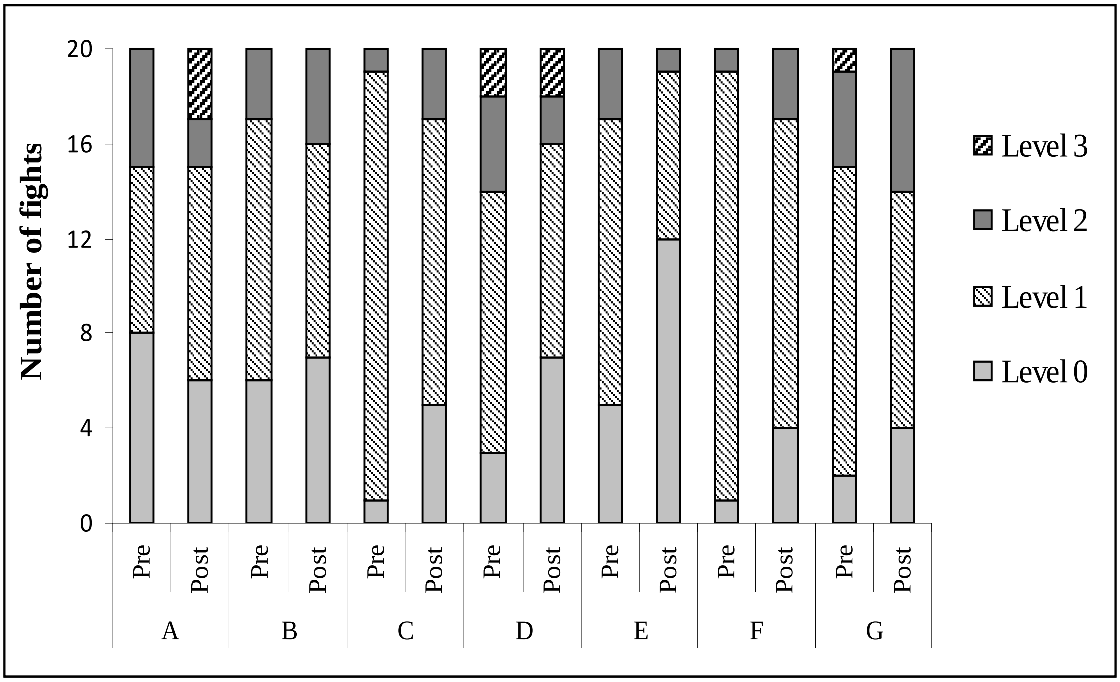


Figure 6.1: The highest levels of aggression reached for each treatment A-G, both pre- and post-treatment. The following treatments of conditioned water were used: A – juvenile, B – sexually mature females, C – inexperienced males, D – new experienced males, E – old experienced males, F – males caring for eggs, G – sea water control.

The highest level of aggression reached for each of the pre-treatment groups A-G was analysed using the Kruskal-Wallis test, with no significant differences observed between the groups ($\chi^2/_2$ = 6.031, d.f. = 6, P = 0.420) indicating that aggression levels were the same between all groups before the conditioned water treatment was applied.

Within each treatment group A-G, the highest level of aggression reached in the pre-treatment trial was compared to that reached in the post-treatment trial using the Wilcoxon Signed Rank test. There were no significant differences found in treatment groups A (juvenile, Z = -1.232, P = 0.218), B (sexually mature females, Z = 0.000, P = 1.000), C (inexperienced males, Z = -0.632, P = 0.527), D (new experienced males, Z = -1.540, P = 0.124), F (males caring for eggs, Z = -0.378, P = 0.705) or G (sea water control, Z = -0.707, P = 0.480). However, a significant difference was found between the pre- and post-treatment levels of aggression with group E (old experienced males, Z = -2.496, P = 0.013). There was a decrease in the number of fights reaching level 1 and level 2 aggression (no level 3 aggression was observed in any fights with this treatment group) and the number of interactions displaying no aggression at all increased.

Although the aggression levels recorded for the pre-treatment groups were compared using two separate statistical tests, the P values obtained have not been corrected for multiple testing. Rice (1989) states that a correction need only be made when two or more tests that cannot be pooled attempt to answer the same null hypothesis. Therefore, as a comparison was only made within each treatment group following the test to compare observed pre-treatment aggression, no correction was made as each test was attempting to answer a different hypothesis.

Female choice following each treatment was noted with the female either selecting the pre-treatment winner or loser of female choice (the loser being the male that was placed in the crystallising dish containing conditioned water). Figure 6.2 below shows the post-treatment female choice (winner or loser) for each treatment of conditioned water used (A-G).

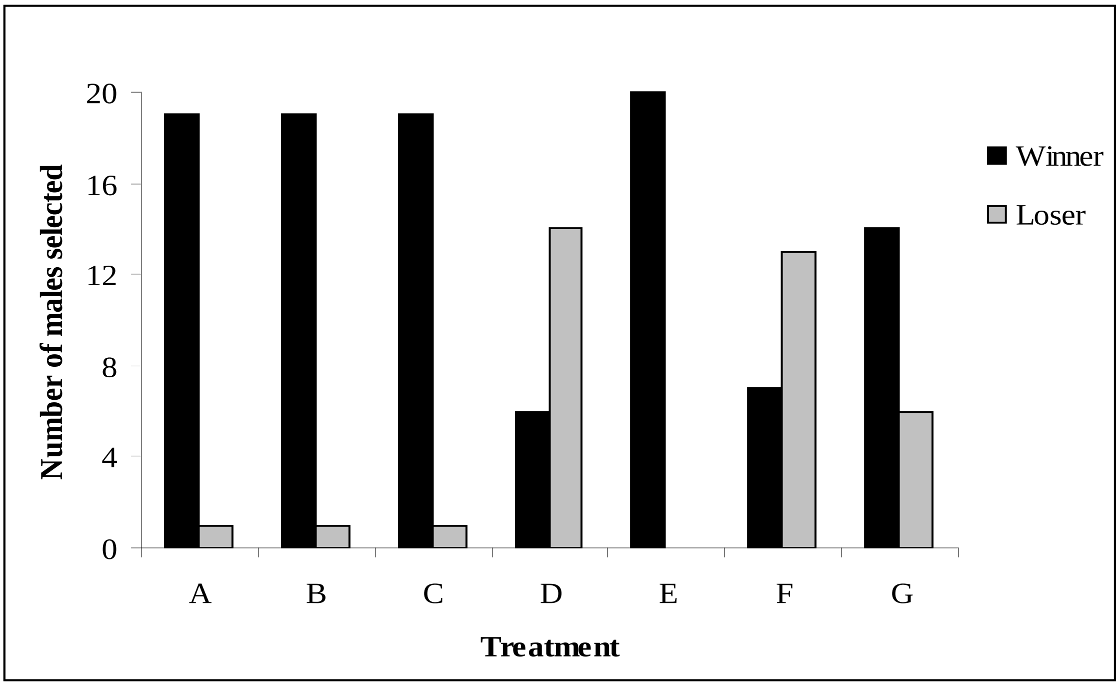


Figure 6.2: The choice of the female following treatment of the loser in conditioned water (female either selected previous winner or conditioned loser), for each of the different treatments. A: juvenile, B: sexually mature female, C: inexperienced male, D: new experienced male, E: old experienced male, F: male caring for eggs and G: sea water control (n = 20 replicates per treatment).

The frequency of female choice for pre-treatment winners and losers following each treatment type was analysed using the Chi-squared test. There was a significant difference observed when the numbers of winners and losers selected in each treatment were compared ($\chi^2/_2$ = 57.286, d.f. = 6, P = 0.000), detailed in Table 6.1 below.

Table 6.1: Contingency table and Chi-squared analysis for each of the different treatments (d.f. = 6).

| Treatment | | Winner | Loser | Total |
|---|---|---|---|---|
| A: juvenile | Observed | 19 | 1 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| B: sexually mature female | Observed | 19 | 1 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| C: inexperienced male | Observed | 19 | 1 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| D: new experienced male | Observed | 6 | 14 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| E: old experienced male | Observed | 20 | 0 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| F: male caring for eggs | Observed | 7 | 13 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| G: sea water control | Observed | 14 | 6 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| | Total | 104 | 36 | 140 |
| | | | $\chi^2/_2$ = | 57.286 |
| | | | P = | 0.000 |

Further to this, the contingency table was subdivided according to Zar (1996) to determine which treatment groups were driving the differences in female choice. For each treatment, the female choice results were compared to the pooled results for all other treatments. It should be noted that Zar (1996) states that such subdividing of tables should only be used as a guide and used to develop additional hypotheses to test on new data sets. However, it does provide an indication of which groups are driving the significant differences. Table 6.2 below shows the subdivision of the contingency table for each of the separated treatment groups tested. As treatment groups A, B and C all have the same frequency of female choice for winners and losers, only one contingency table has been performed for these values.

Table 6.2: Contingency tables and Chi-squared analysis of the subdivided treatment groups A-G compared to the pooled data of the other groups (A: juvenile, B: sexually mature female, C: inexperienced male, D: new experienced male, E: old experienced male, f: male caring for eggs and G: sea water control) (d.f. = 1 in all cases).

| Treatment | | Winner | Loser | Total |
|---|---|---|---|---|
| A/B/C | Observed | 19 | 1 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| All other treatments | Observed | 85 | 35 | 120 |
| | Expected | 89.1 | 30.9 | 120 |
| | Total | 104 | 36 | 140 |
| | | | $\chi^{2}/_{2}$ = | 5.241 |
| | | | P = | 0.022 |
| D | Observed | 6 | 14 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| All other treatments | Observed | 98 | 22 | 120 |
| | Expected | 89.1 | 30.9 | 120 |
| | Total | 104 | 36 | 140 |
| | | | $\chi^{2}/_{2}$ = | 23.957 |
| | | | P = | 0.000 |
| E | Observed | 20 | 0 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| All other treatments | Observed | 84 | 36 | 120 |
| | Expected | 89.1 | 30.9 | 120 |
| | Total | 104 | 36 | 140 |
| | | | $\chi^{2}/_{2}$ = | 8.077 |
| | | | P = | 0.004 |
| F | Observed | 7 | 13 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| All other treatments | Observed | 97 | 23 | 120 |
| | Expected | 89.1 | 30.9 | 120 |
| | Total | 104 | 36 | 140 |
| | | | $\chi^{2}/_{2}$ = | 18.852 |
| | | | P = | 0.000 |
| G | Observed | 14 | 6 | 20 |
| | Expected | 14.9 | 5.1 | 20 |
| All other treatments | Observed | 90 | 30 | 120 |
| | Expected | 89.1 | 30.9 | 120 |
| | Total | 104 | 36 | 140 |
| | | | $\chi^{2}/_{2}$ = | 0.224 |
| | | | P = | 0.636 |

Therefore, all groups apart from the sea water control are significantly different to the expected frequencies of female choice for winners and losers. For the treatment groups A, B, C and E, females chose the previous winner in more cases than were expected. For the treatment groups D and F, females chose the previous loser in more cases than were expected. For treatment group G, the sea water control, the expected frequencies of previous winners and losers were observed.

## 6.4: Discussion

The results indicate that the pre-treatment aggression levels were not significantly different between the treatment groups (Figure 6.1). The highest aggression level was recorded between two inexperienced males, before any conditioned water was applied so individuals in every trial were expected to behave in a similar way. Following the separation phase where different treatments were applied, aggression levels did not significantly change, apart from with treatment group E: old experienced males. Following the use of conditioned water from old experienced males, the aggression was observed to decrease. Following treatment with old experienced male water, more fights showed no aggression compared to the pre-treatment results. Also, the occurrence of fights reaching level 1 and level 2 decreased (there were no incidences of level 3 aggression either pre- or post-treatment). This was the only treatment group to show significant differences in aggression, although some decreases in aggression were observed following conditioning with new experienced males and males caring for eggs. This change therefore must be due to the conditioned water. It may be that old experienced males are more likely to win aggressive fights between individuals and the opponent of the male placed in conditioned water from this source could detect this. Age-related dominance has been shown demonstrated in the African elephant *Loxodonta africana*, where older males have heightened testosterone levels leading to heightened aggressive displays (Hollister-Smith *et al*., 2007). It is possible that the water from old conditioned males reflects this type of heightened dominance to the other male. Another possible explanation is that old males are larger in size than new experienced males as they have undergone a further two weeks of growth and this size difference may be conferred chemically to the inexperienced opponent. Individuals assess the Resource Holding Potential (RHP) of their opponent and base decisions on this assessment whether to escalate on this assessment (Sneddon *et al*., 1997). If a size difference is conveyed to the opponent, the fight may not escalate as that individual recognises that a size difference is evident and the fight will not escalate (Maynard Smith, 1974). Smaller males may avoid conflict by assessing themselves to be smaller than their opponent (Turner, 1994) and this may explain why aggression decreased following treatment with water conditioned from an old experienced male.

Conditioned water has been shown to have an effect on the behaviour of individuals in kin recognition trials (Gerlach and Lysiak, 2006), predator recognition trials (Mann *et al*., 1984) and in female choice trials (Barata *et al*., 2008). Females have been shown to respond in Y-maze trials to water conditioned by sexually receptive males but not to water conditioned by sexually receptive females in the blenny *Salaria pavo* (Barata *et al*., 2008). With *N. acuminata*, placing individuals in conditioned water from some sources did have an effect on subsequent female choice (Figure 6.2). Female choice frequency for pre-treatment winners and losers was found to be significantly different for all treatment groups apart from with the sea water control treatment. As there was no significant change in female choice when losers were placed in the sea water control treatment, separating the individuals does not affect the female's subsequent choice of individual and the female is more likely to select the male chosen in the first interaction. For the treatment groups A, B, C and E, females chose the previous winner in more cases than were expected. Females selected significantly more winners for treatment groups A: juvenile, B: sexually mature female, C: inexperienced male and E: old experienced male. It is possible that placing the loser in conditioned water from these sources reinforced the female's choice of previous winner.

Conditioned water from both new experienced males (D) and males caring for eggs (F) had a significant effect on the subsequent choice of the female. With both of these treatments, female choice following separation changed and the pre-separation loser was selected over the previous winner of female choice. It is possible that there is a change in the male that occurs during egg care that makes the male preferable to females. Male parental care ability in *N. acuminata* may be expressed chemically to the female following successful egg care. For example, prolactin has been found to be related to experience of parental care (Smith and Hoar, 1967). In the common marmoset *Callithrix jacchus*, the levels of prolactin vary significantly between males carrying infants and non-parental males (da Silva Mota *et al*., 2006) and prolactin levels also vary between first time fathers and those that have successfully reared a brood (Schradin and Anzenberger, 2004).

There was no significant change in female choice when loser males were placed in conditioned water from old experienced males. It may be that once males have completed egg care, the scent of experience wears off, as the 'scent of experience' comes from the eggs rather then the male. However, if this were the case, it would be unlikely that conditioned water from new experienced males would have an effect on altering female choice following masking of the loser. It is possible that as males increase in age, there is a decrease in their attractiveness to females. Female choice related to age of males has been discussed in the literature with hypotheses suggested both supporting and opposing female choice for older males. A male may show he has good genes merely by survivorship (Manning, 1985); older males show higher genetic quality for viability (Brooks and Kemp, 2001). Older males may also invest more in offspring and be better parents (Brooks and Kemp, 2001). Features of male behaviour may also develop with age (Manning, 1985). In. *N. acuminata*, the jaw increases in size relative to the growth of the individual (Reish and Alosi, 1968), therefore the weapons used for aggression and defence of offspring increase with increasing age, with the potential for a older and therefore larger male to be better at defending his eggs mass. In the fruit fly *Drosophila melanogaster*, older males had higher reproductive success than younger males due to the longer copulation time and greater volume of sperm transferred by older males (Avent *et al*., 2008). However, old males should be discriminated against if aging reduces their reproductive fitness (Manning, 1985) and older males are less capable of producing high quality sperm (Brooks and Kemp, 2001). The older the male, the greater the chance that mutations have accumulated that potentially offset the advantages of survivorship (Crow, 1993). Younger males may also be better adapted to the present environmental conditions than older males (Brooks and Kemp, 2001). In the sandfly *Lutzomyia longipalis*, females discriminate against older males which have reduced hatching success than younger males; females benefit from increased fitness by avoiding poor quality mates (Jones *et al*., 2000). Pervez and Richmond (2004) found that in the ladybird beetle *Propylea dissecta*, females selected intermediate aged males that produced more viable offspring than either younger of older males. A similar selection for intermediate-aged males was observed in the pigeon *Columba livia*, mates were preferred if they were experienced but avoided if they were over a certain age (Burley and Moran, 1979). It is possible that in *N. acuminata*, there is an optimum age of male; a male that is experienced in parental care but not too old to provide adequate care or maximum reproductive success.

There does appear to be an effect on female choice linked to males are involved in egg care but also when males that are not currently caring for an egg mass but have recently completed egg care. There is evidence that conditioned water from these sources has an effect on female mate choice but as there was no significant difference between the change in female choice for treatments with males caring for eggs and new experienced males, there is no further evidence as yet to help identify the source of the scent of experience. During egg care, there may be a physiological change in the male after sperm release, after the eggs have hatched or following the production of more sperm. Very little is known about the physiology of males in terms of reproduction in this species. The scent of experience may just emanate from the smell of eggs, as conditioned water from both males caring for eggs and new experienced males altered the subsequent choice of the female. As females were only given a choice of two males, one inexperienced, and one from conditioned water, the female may select the male that is the 'best case scenario', the male that smells of eggs. A further treatment group using conditioned water from egg masses may have shed light on whether the female was selecting for the smell of eggs or for the smell of the experienced male. However, if females were selecting for the smell of eggs alone, it would be unlikely that the conditioned water from new experienced males would have had an effect on female choice. In these experiments, previous losers placed in conditioned water from new experienced males were selected over the previous winner in 14/20 cases.

In summary, conditioned water does not appear to affect the aggression levels observed between two males, except when the loser is placed in conditioned water from an old experienced male. This may be due to the other male recognising the opponent as experienced and with a better chance of winning a fight but this is unlikely as this was not observed when losers were placed in conditioned water from new experienced males. It is more likely that a size difference is conveyed in the conditioned water from old experienced males. When loser males were placed in conditioned water from old males, the female chose the previous winner and did not select the conditioned loser. It is possible therefore that a male may be considered too old to undertake parental care and is no longer preferred over an inexperienced male. Female choice was observed to change from previous winners to losers placed in conditioned water from both new experienced males and from males caring for eggs. This provides further evidence that females can detect males as experienced through chemical signals as this was the only variable changed during the experiments, but does not provide further evidence as to the mechanism involved or the origin of the 'scent of experience'.

### 6.4.1: Next steps

Further investigations need to be undertaken to look at the behaviour of a female towards older experienced males and what effect this has on female choice. There may be that there is a point where males become too old to undertake parental care and are no longer preferred by females over inexperienced males, possibly due to their physical condition. The scent of experience also needs to be investigated further using a greater number of replicates to learn more about the possible chemical signal released. Future trials should include replicates involving egg masses without a male present to determine what effects a male conditioned with water surrounding egg masses would have on a female and the length of time any effect may have. Conditioned water should also be tested from males that have not completed egg care to determine the effects of this on female choice. The potential physical changes in the male following parental care also need to be investigated to understand further its importance in female choice.

# Chapter Seven:

# The occurrence of male parental care for eggs fertilised by a different male

## 7.1: Experimental reasoning and aims

Adoption is defined as the care of offspring by individuals that are not the genetic parents (Wilson, 1975 cited Riedman, 1982) and has been reported in fish, bird and mammal species (Riedman, 1982). However, it may be difficult to define what is full adoption, rather than just social interaction, as assistance may be given to the parents but offspring will not be fully cared for by the foster parent (Wisenden, 1999). Alloparental care is another form of tending to offspring but differs from adoption as the genetic parent may return to care for their progeny (Riedman, 1982). With adoption however, full care of the offspring is assumed with no consequent care from the genetic parents. Therefore, levels of care constituting adoption are hard to define and can vary from egg defence through to feeding (Meek and Robertson, 1991). Both adoption and alloparental care are hypothesised to be reproductively costly and inconsistent with classic evolutionary theory (Riedman and Le Boeuf, 1982) but the benefits of adoption may offset such costs incurred (Brown, 1998). During adoption, parental behaviours may be transferred to offspring through imprinting and may include preferences for food, mate choice, a particular niche preference or foraging techniques (Avital *et al*., 1998).

Debate has emerged as to the reasons why individuals may undertake such a seemingly costly act of parental care and the following theories have been proposed to attempt to explain the occurrence of adoption and alloparental care: altruistic behaviour, selfish behaviour, kin selection, acts of reciprocity, misdirected acts (or mistakes) and forced acts (Daniels, 1979).

Adoption may form a true altruistic act with no selfish benefits to the carer, as observed in social insects such as termites, wasps, bees and ants where sterile castes exist that care for offspring of the queen (Wilson, 1975). However, such unselfish behaviour is rare and it is more likely that the carer will receive benefits in some way, making the act selfish. Such benefits may include receiving an increased number of matings due to caring for offspring, enjoying increased social status, increased access to resources such as food or shelter, gaining parental experience and enhancing parenting skills (see Daniels, 1979 for review). Caring for young may influence the future reproductive success of the male (Riedman and Le Boeuf, 1982) and may be favoured by natural selection to enhance the adoptive individuals parenting skills (Riedman and Le Boeuf, 1982). This is especially important for species where competent parenting skills are vital for example if females produce a very limited number of eggs (Riedman, 1982). In the elephant seal *Mirounga angustirostris*, females without offspring may care for pups to gain experience. Pup mortality is high with inexperienced mothers and as females can only produce a limited number of seals per lifetime, care of such pups is important (Riedman and Le Boeuf, 1982). Such behaviour by females to gain parental experience has also been observed in chimpanzees, rhesus macaques and gorillas (Riedman, 1982). In fish species, males have been shown to care for eggs not their own as this influences females to spawn in a nest that contains eggs, rather than empty nests (Constanz, 1985) and females have been shown to copy the previous mate choice of another female (Wisenden, 1999). This has been observed in the fathead minnow *Pimephales promelas*, where females are attracted to males with eggs at nests sites. Adopted eggs however receive less care and have increased mortality rates and only the minimum number of eggs required to attract a mate are maintained in the nest (Sargent, 1989). Other examples of males that use eggs to attract mates have been observed in the pygmy sculpin *Cottus pygmaeus* (Johnston, 2000), the fathead minnow *Pimephales promelas* (Unger and Sargent, 1988) and the three-spined stickleback *Gasterosteus aculeatus* and males have been observed to kidnap eggs from other nests to achieve matings (Ridley and Rechten, 1981). One further example of a male fish that exploits this female preference for eggs in nests is the fantail darter *Etheostoma flabellare* and as such, males of this species have developed two strategies to influence females: adoption (Rohwer, 1978) and egg mimicry. The males possess dorsal spines with a fleshy ball coloured orange with a black rim with a transparent membrane at the tip. When the male is positioned near the roof of the nest, these balls closely resemble an egg mass. Such egg mimics were found to be preferred over males that did not have an egg mass in the nest or possess such appendages (Knapp and Sargent, 1989). Tallamy (2000) postulates in arthropods that if the presence of an egg mass increases the attractiveness of the male, unrelated eggs should be accepted by the male but also competed for against other males. Therefore, males should be willing to care for unrelated eggs if such care attracts additional mates and therefore increases reproductive success (Tallamy, 2000).

Caring for related offspring may be a form of kin selection (Daniels, 1979), aiding a closely related individual and therefore influencing its own genetic fitness (Riedman, 1982). This has been observed in the cichlid *Lamprologus brichardii* where helpers aid in the defence and feeding of closely related siblings (Taborsky and Limberger, 1981).

Older juveniles of the monkey species *Callithrix jacchus* partake in young carrying, a form of parental care, of related siblings (Box, 1977). In social insects, such as wasps, bees, ants and termites, sterile workers in the colony care for the offspring of a single laying queen, which includes constructing and protecting the nest, foraging for food and tending for the larvae (Wilson, 1975). Workers do not produce any offspring of their own so have no personal fitness but their actions greatly improve the reproductive efforts of the queen (Sudd and Franks, 1987).

Individuals may also assume parental care roles if it is an act of reciprocity; if the favour is returned later, for example where alloparents receive help with their offspring in turn (Daniels, 1979). In the cichlid *Tilapia rendalli*, four parents care for two brood sets which may lower the risk of predation to both sets of offspring (Ribbink *et al.*, 1981). In harsh or stressful environments where breeding resources such as food and space may be limited, parental cooperation may be beneficial to both parties (Riedman, 1982). Males of the cichlid *Cichlasoma nicaraguense* have been observed to defend the young of the predator cichlid *Cichlasoma dovii*. It is thought that this is an act of reciprocity as the predator *C. dovii* will feed on the main competitors of *C. nicaraguense* (McKaye, 1977). However, this has been contested by Coyne and Sohn (1978) who suggested that the findings may be a case of mistaken identity on the part of *C. nicaraguense* individuals.

The act of adoption may occur as a result of a mistake. Mistaken identity and subsequent brood mixing may occur if parents are unable to recognise their own offspring (Daniels, 1979; Silk, 1999). Such instances of brood mixing have been observed in the cichlids *Pseudocrenilabrus multicolour* (Mrowka, 1987), *Haplochromis polystigma* (Ribbink, 1977) and *Hemichromis bimaculatus* (Greenberg, 1961), to name a few examples. However, parental care may also be forced upon the individual. If parents cannot recognise their own offspring, other parents may place offspring with such individuals so as to avoid parental care duties themselves, known as brood parasitism or cuckoldry, where eggs are laid in the nests of other individuals (Weller, 1959). Such behaviour has been observed in a number of fish, bird and insect species, such as the minnow *Pungtungia herzi* that deposit eggs in the nests of the freshwater perch *Siniperca kawamebari* (Baba *et al.*, 1990), the barnacle goose *Branta leucopsis* that lay eggs in the nests of conspecifics (Anderholm *et al.*, 2009) and the lace bug *Gargaphia solani* that lay eggs among the egg masses of conspecifics (Tallamy, 1985).

The classic example of cuckoldry is found in the cuckoo *Cuculus canorus* where females lays eggs in a host's nest, such as the meadow pipit *Anthis pratensis*, and removes one or more of the host species' eggs (Lack, 1963). Such parasitism benefits the genetic parents as the offspring will carry their genetic material, but the parents do not incur the costs associated with parental care (Wisenden and Keenleyside, 1992). However, in some species, this may still provide a benefit to the adoptive parent as an increased number of offspring equals a dilution of predation risk (Smith, 1968 cited McKaye *et al.*, 1992). Not only does egg survival increase with the number of eggs present, but foreign young accepted into an existing fry group may aid differential predation so smaller young may be accepted that are more likely to be preyed upon (Smith, 1968 cited McKaye *et al.*, 1992). Such young are often kept on the periphery and "nipped back" if they stray into the centre of the fry group (McKaye *et al.*, 1992). Such behaviour has been observed in the convict cichlid *Cichlasoma nigrofasciatum* (Fraser and Keenleyside, 1995) and the catfish *Bagrus maridionalis* (McKaye *et al.*, 1992).

Males may acquire non-descendent young by evicting a resident male from a spawning territory or nest site already containing eggs to enhance his attractiveness to females. They then guard and care for old and new eggs. However, the fathead minnow *Pimephales promelas* prefers to cannibalise non-descendent eggs once the male has mated with a female (Wisenden, 1999). Large and dominant peacock wrasse males (*Symphodus tinca*) have also been observed taking over a nest site to spawn and then leaving the original male to care for the brood. The large male therefore incurs no costs of parental care (nest maintenance, guarding) and the original male is unlikely to abandon the nest site if this also means abandoning his brood as well (van den Berghe, 1988). Kidnapping of broods also occurs, for example in the cichlid *Cichlasoma citrinellum* and it is hypothesised in this species that this dilutes the predation risk for the individual's own brood (McKaye and McKaye, 1977).

Due to parental death or abandonment, *N. acuminata* eggs normally under parental care may be found uncared for in natural populations, although this is rare as males caring for eggs hardly leave the egg mass, except to forage at the mouth of the burrow (Reish, 1957). Approximately 10-15% of males abandon the parental tube containing developing eggs (Reish, personal communication). Eggs left in a tube will not survive longer than two days (Reish, 1980a) and will turn a white colour when dead (personal observations). Initial studies have shown that *N. acuminata* males will take care of eggs that are not their own. A preliminary study by Sutton (1998) found that males would care for eggs of another male after parental males have been removed from the burrow and replaced. In the study by Sutton (1998), males were reported to have adopted eggs if two distinct behaviours are observed: egg fanning to oxygenate the eggs and aggression towards potential mates (Sutton, 1998). These criteria concur with the original work by Reish (1957), where males undertake fanning behaviour to oxygenate the egg mass and fight both male and female intruders to the parental burrow. Replacement males will undertake parental care following the paternal male death and it was hypothesised that inexperienced males would undertake such care in order to gain ‘experience’ and subsequently be preferred by females in future reproductive events (Reish, personal communication). Females of this species have been shown to preferentially select males that have previously undertaken egg care compared to ‘virgin’ males (Fletcher *et al.*, 2009) and therefore may select mates on the basis of their parental care abilities. The completion of egg care also signals to the female that the male is likely to be of high quality as he has survived to produce offspring despite the high costs associated with egg care (Tallamy, 2000). It is unknown, however, whether such adoption and selection for experienced males occurs in the wild.

The aim of this chapter was to determine if care of eggs from another male occurs under experimental conditions in the laboratory and if there was a difference in the behaviour of experienced males (those that have previously undertaken egg care) and inexperienced males. It was hypothesised that care of eggs fertilised by another inexperienced male occurs under experimental conditions and also that experienced males will not care for eggs that they have not fertilised. It was hypothesised that experienced males gain no benefit from such behaviour whereas inexperienced males may benefit by increasing their parental experience. In this study, cannibalism was also noted if it occurred. As previously mentioned, cannibalism of eggs has been known to occur in this species (Oshida *et al.*, 1981) and it seems unlikely that a male would leave an egg mass (a potential food source) if it will just be consumed by a competitor. A *N. acuminata* male encountering an abandoned egg mass was observed cannibalising them (Starczak, 1984). Males undertaking cannibalism of eggs would likely do so to gain nutritional benefits and do not care for the eggs so they develop.

## 7.2: Methodology

A male and female from the Reish population maintained in the laboratory were paired in a crystallising dish (70mm diameter) containing 50ml of filtered sea water (36-38‰) to produce fertilised eggs. During pairing behaviour and before egg release, the crystallising dish was placed in an aerated container (180mm x 100mm x 140mm) with approximately 2 litres of sea water (36-38‰) to minimise disturbance and to regulate the environmental conditions. Animals were fed a small amount of low protein rabbit food (non-processed compressed hay, Smith's Animal and Pet Supplies, Castle Road, Hull) *ad libitum* throughout the experiment. Eggs were shed in the parental tube by the female and fertilised by the male. After the first division of the eggs was observed, the parental male was gently removed from the parental tube by placing a paintbrush at the tail end of the animal; the male reluctantly vacates the parental tube in response to this stimulus, leaving the egg mass intact. The parental male was then replaced with another sexually mature male, also from the Reish population. The subsequent behaviour of the replacement male was observed using the scoring system detailed in Table 7.1 below. Egg fanning was used as a good indication that introduced males were undertaking parental care. Any cannibalism was also noted, confirmed by observation of eggs in the gut. Introduced males were scored after 5 minutes, after 24 hours and after 48 hours. After 48 hours, if the male was still present with the eggs in the parental tube, observations of the egg mass were made every day to determine if the eggs hatched.

Table 7.1: Scoring system for parental and replacement male behaviour.

| Score | Observation |
|---|---|
| 0 | Male entered tube and fanned eggs |
| 1 | Male ignored eggs and constructed a new tube |
| 2 | Male cannibalised eggs |

Two different categories of males were introduced into the crystallising dish (70mm diameter) containing the egg mass, depending on their reproductive history: experienced males (n = 6) and inexperienced males (n = 6). Inexperienced males were separated from the population at the larval stage and allowed to mature sexually but prevented from reproducing with a female. Experienced males were allowed to reproduce once with a female (males were previously inexperienced males as above) so will have already successfully cared for eggs that hatched. To ascertain that males would enter constructed tubes and proceed to fan eggs, parental males were removed from the burrow, isolated for ten minutes and then re-introduced and scored as per above (n = 15).  The total number of replicates for this study equalled 27.

7.3: Results

When a male was introduced to a crystallising dish containing an egg mass within a burrow, the ensuing behaviour was found to vary depending on the reproductive state of the male, as shown in Figure 7.1 below. Parental males were found to return to their burrows and care for eggs, after a period of swimming behaviour. All but one of the fifteen re-introduced parental males successfully produced eggs that hatched; one brood was cannibalised. When inexperienced males were introduced into the crystallising dish with an egg mass, 5 out of 6 males fanned the eggs and emergent juveniles were observed following hatching. One male cannibalised the egg mass. When experienced males were introduced into the crystallising dish with an eggs mass, none of the males were observed to fan the eggs and three of the males cannibalised the egg mass. The other three males ignored the egg mass and constructed a new burrow in the same crystallising dish.

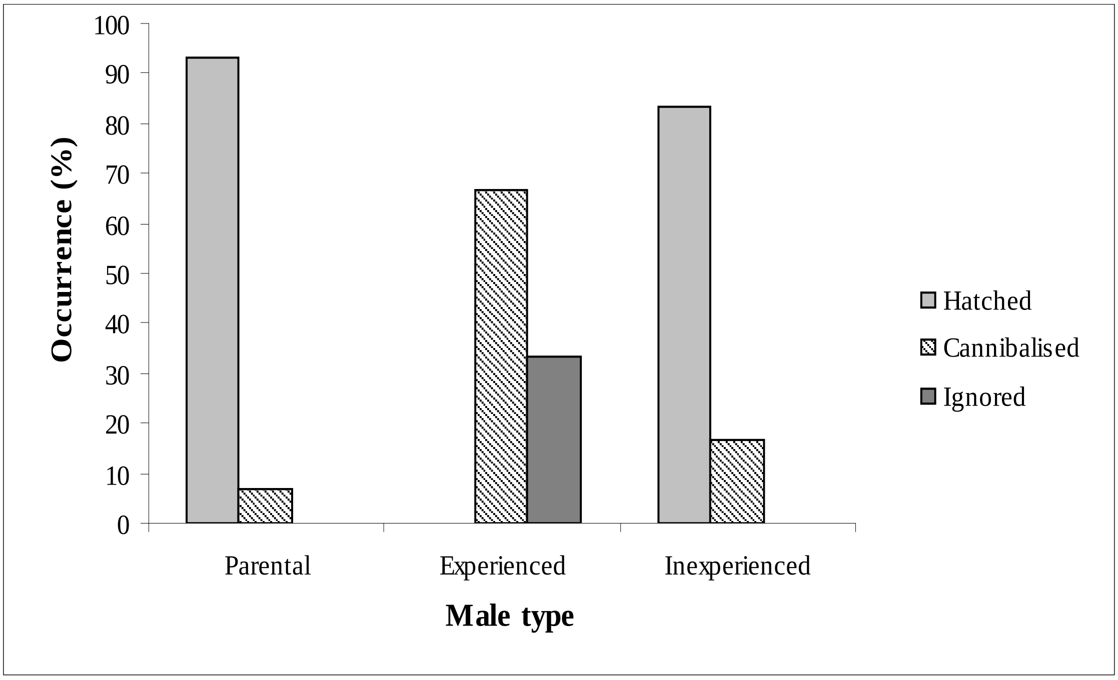


Figure 7.1: Percentage of eggs hatched, cannibalised or ignored by males from the three groups: re-introduced parental males, experienced males and inexperienced males.

The differences between hatched, cannibalised and ignored eggs for each type of male were analysed using the Freeman-Haltman extension of the Fisher's exact test for a 3 x 3 contingency table (expected frequencies were < 5). There was a significant difference found between the frequencies of eggs masses that were hatched, cannibalised or ignored (Fisher's exact = 0.000). Further to this, the contingency table was subdivided according to Zar (1996) and Fisher's Exact tests used to determine the location of the differences in behaviour between the three types of male used. This can only be used as a guide but will give an indication of which data set is driving the significant difference (Zar, 1996). Table 7.2 below shows the subdivision of the results with pooled data for both ignored and cannibalised eggs due to the low frequencies of ignored egg masses.

Table 7.2: Subdivision of the contingency table with each type of male used: parental, experienced or inexperienced.

| Male type | | Hatched | Other | Total |
|---|---|---|---|---|
| Parental | Observed | 14 | 1 | 15 |
| | Expected | 10.3 | 4.4 | 15 |
| Non-parental | Observed | 5 | 7 | 12 |
| | Expected | 8.4 | 3.6 | 12 |
| | Total | 19 | 8 | 27 |
| | | | **Fisher’s** = | 0.008 |

| | | | | |
|---|---|---|---|---|
| Experienced | Observed | 0 | 6 | 6 |
| | Expected | 4.2 | 1.8 | 6 |
| Non-experienced | Observed | 19 | 2 | 21 |
| | Expected | 14.8 | 6.2 | 21 |
| | Total | 19 | 8 | 27 |
| | | | **Fisher’s** = | 0.000 |

| | | | | |
|---|---|---|---|---|
| Inexperienced | Observed | 5 | 1 | 6 |
| | Expected | 5.4 | 0.6 | 6 |
| Parental | Observed | 14 | 1 | 15 |
| | Expected | 13.6 | 1.4 | 15 |
| | Total | 19 | 2 | 21 |
| | | | **Fisher’s** = | 0.500 |

There was a significant difference in the behaviour of the parental male compared to the pooled results from the inexperienced and the experienced male (P = 0.008). However, there was no significant difference found between the behaviour of parental males to inexperienced males towards an egg mass (P = 0.500). However there was a significant difference in behaviour when experienced males were compared to the pooled results from parental and inexperienced males (P = 0.000). Therefore, although the results from the parental type male were significant to the other types of male, the behaviour of the experienced males towards egg masses is driving the significant difference.

## 7.4: Discussion

Several theories have been suggested as to why individuals would adopt and care for young not their own: altruistic behaviour, selfish behaviour, kin selection, acts of reciprocity, misdirected acts/mistake and forced acts (Daniels, 1979). The associated benefits related to caring for such offspring, such as increased reproductive success (Tallamy, 2000) and increased social status (Daniels, 1979) have also been proposed.

In this study, inexperienced males were found to care for eggs not their own (apart from in one case where the eggs were ignored). Experienced males were observed either ignoring or cannibalising egg masses (Figure 7.1). However, these results should be viewed with caution as only six inexperienced and six experienced males were introduced. During the undertaking of these experiments, several variables were not controlled prior to the experiment. As the Reish population is likely to be a heavily inbred population, with only six individuals collected in 1964, this may affect any results observed with males caring for eggs not fertilised by them. As individuals are likely to be closely related genetically, males may care for such eggs in order to pass on their genes. Individuals may also have originated from the same brood as the parental male, although an attempt was made to control this by separating individuals at the larval stage and noting the brood they originated from. Older siblings have been shown to help care for brothers and sisters, as in the monkey *Callithrix jacchus*, where older juveniles partake in young carrying of younger siblings, a form of parental care (Box, 1977). In this study, the complete parental lineage and the relatedness of the individuals used were unknown. However, if males were caring for eggs due to their genetic relatedness, it would be expected that experienced males would also care for eggs; this was not observed in these experiments.

Two studies have observed the caring behaviour of cichlid fish and found that the previous experience of the males did not affect adoption as both experienced and inexperienced males adopted young. The cichlids *Tilapia sparrmani* and *Aequidens partalegrensis* both accepted young of the other species (Collins and Braddock, 1962) as did the cichlids *Hemichromis bimaculatus* and *Aequidens partalegrensis* and both experienced and inexperienced males raised successful broods (Greenberg, 1961). However, in these species, young were only accepted when males had broods themselves and accepted young were similar in age to those broods. It may be the case that in *N. acuminata*, males only recognise broods as their own by their physical size.

As inexperienced males undertook care of eggs but experienced males did not, it does not seem likely that care occurs due to a mistake in the recognition of offspring. If males cannot recognise their own offspring, then experienced males would also be expected to undertake parental care of eggs if introduced to an egg mass. The other theories proposed for such care also do not appear to relate to the observed results. If caring for eggs from another male were an altruistic act or an act of reciprocity, then it would be expected again that both inexperienced males and experienced males would care for such eggs. Caring for such eggs is also not a forced act as males were put at the entrance of a burrow already containing eggs rather than eggs added to their own burrow. Due to the documented life cycle in this species of male and female monogamous pairing, female dehiscence and male parental care (Reish, 1957), it seems unlikely that cuckoldry occurs in this species. It may be the case, however, that *N. acuminata* males abandon their eggs to be cared for by another male. This ensures that the parent's genetic information is passed on but the male incurs no costs of care (Wisenden and Keenleyside, 1992). Such behaviour has been shown to occur in fish, such as the minnow *Pungtungia herzi* where parents 'dump' eggs in another individual's nest. This also seems unlikely in *N. acuminata* as there would be a risk that the abandoned eggs would either be cannibalised by a different individual (either male or female) or the eggs would die due to a lack of oxygenation. This occurs after 2-3 days if eggs are not fanned by the male (Reish, 1980b).

Experienced males may not have cared for the eggs because they were of poor quality (diseased or dead). Consuming such eggs may prevent the spread of infection and protect the remaining eggs (Kraak, 1996). In three instances where an experienced male was introduced, the entire egg mass was consumed by the experienced male when cannibalism was observed. Also, cannibalism was only observed in one instance with inexperienced males (Figure 7.1). It seems unlikely that experienced males were placed with poor quality eggs in each trial but the inexperienced male was not. However, more replicates would be needed to reduce the likelihood of poor quality eggs affecting the results.

When offspring require prolonged or extensive parental care, competent parental skills may be an important factor affecting reproductive success (Riedman, 1982). Inexperienced males may benefit by caring for eggs not their own by gaining parental experience and an increase in reproductive success, a form of selfish behaviour. If the ‘scent of experience’, as examined previously in Chapter 6, is indeed how parental care ability is conferred to the female, this signal should be an honest and reliable indicator of ability (Holezer, 1989). If males can gain an advantage by caring for males not their own then there should be associated costs to such behaviour in order for the signal of parental care experience to be an honest indicator for the female (Hoelzer, 1989). Adoption may cost males if they have to care for eggs even for only a short amount of time. Costly male behaviour for the purpose of obtaining a mate, however, is not unusual (Härdling and Kaitila, 2004). If the female cannot detect that males have not successfully produced (or cared for) viable offspring, this signal may not be an honest indication to the female and parental experience may be expressed to the female in some other way, for example via physical condition. If females can tell the difference between cannibals and non-cannibals and males that only care for eggs for a short time period, then adoption may not be cheating. This needs further investigation as the results obtained in Chapter 5 (female detection of cannibalistic males) were inconclusive.

Male parental care ability may also be expressed to the female chemically following successful egg care. A relationship has been found with the expression of prolactin and parental care (Smith and Hoar, 1967). In birds and mammals, prolactin is known to be involved in maternal behaviour (Schradin and Anzenberger, 2004) but in some mammal and fish species, increases in prolactin correspond with male parental care (Nunes *et al*., 2000), as observed in the common marmoset *Callithrix jacchus* where the levels of prolactin vary significantly between males carrying infants and non-parental males (da Silva Mota *et al*., 2006). There may also be a relationship between prolactin levels and experience of males, for example differences between first time fathers and those that have successfully reared a brood (Schradin and Anzenberger, 2004). Similarly, in the sex-changing goby *Lythrypnus dalli*, experienced males were found to have higher ketotestosterone levels than inexperienced males (Rodgers *et al*., 2006). It may be the case in *N. acuminata* that experienced males signal to the female that they are capable of providing good parental care but the processes involved in the production and recognition of such a cue in *N. acuminata* are still unclear.

In summary, previous work in *N. acuminata* and observations made in this study indicate that there is a difference between experienced and inexperienced males when it comes to adopting egg masses. Parental males will also return to their burrows if separated from the egg mass. The replicates undertaken here did not take into account a series of variables that would have been important in forming the basis of these hypotheses and as such, these experiments should be repeated, taking such variables into account. It is clear that further work needs to be undertaken to disentangle the relationships between parental care, experience and female mate choice in this species.

### 7.4.1: Next steps

It is apparent that the experiments undertaken in this chapter need to be repeated, taking into account the relatedness of the individuals within a population, requiring careful notation of family lineages and relatedness of offspring and ‘carers’ but also taking into account the quality and the number of eggs laid. It would also be of use to determine if such behaviour involving caring for eggs occurs in any of the other populations maintained in the laboratory or if such care occurs in wild populations or even within mesocosm (a larger population tank housed in the laboratory). It would be interesting to note the reaction of the introduced male caring for an egg mass when a female is placed in the same crystallising dish. The parental male would defend the eggs and not leave the parental burrow but would the same reaction be observed with an introduced male? Observing a parental male’s behaviour following the addition of another male’s eggs to the egg mass that he is caring for may also give an indication if individuals can recognise their own offspring in *N. acuminata*. Following a male completing care of eggs that he did not fertilise, experiments involving female choice tests would be useful to determine if the male with new parental experience is now preferred over an inexperienced male and if males have to care for eggs until they hatch. It may be that males only need to have the smell of eggs for them to be preferred by a female, since it is still unclear if males can recognise their own egg mass so further experiments are needed to investigate if males have a preference for their own egg mass.

# Chapter Eight:

# Final discussion and conclusions

Reproduction in *N. acuminata* is unique among nereid polychaetes (Bridges *et al.*, 1996). A male and a female form a monogamous pair bond and occupy the same mucous burrow (Reish, 1957). Once females have reached sexual maturity, they lay their eggs in the burrow and die (Reish, 1957). Males undertake sole care of the offspring and once larvae vacate the parental burrow, the male can go on to reproduce again (Reish, 1957). The populations of *N. acuminata* maintained in the laboratory all share this unique reproductive cycle and share similar morphological traits (Pettibone, 1963; Weinberg *et al.*, 1990). There is evidence however, that these populations have diverged and form a species complex.

Species can be separated by changing land masses for example and separated species may continue to evolve from each other independently and diverge from each other with time (Ribinoff and Rubinoff, 1971). Sibling species are morphologically similar, if not identical populations that are reproductively isolated (Mayr, 1977), making it difficult to establish which populations are still conspecific (Lovern *et al.*, 1999). Biotic and abiotic factors of the environment can shape the individuals that occupy that habitat. Communication systems used by individuals in populations can also be adapted to changes in the environment (Lovern *et al.*, 1999). Differences in temperature, humidity, salinity, level of predation, food availability parasite prevalence and other factors of the habitat conditions may favour differences in sensory systems which in turn select for differences in signal design (Mayr, 1977; Madden, 2006; Ward and McLennan, 2008). Alternatively, differences in habitat noise and transmission properties may favour differences in signal design even if there is no change in the sensory system (Leal and Fleishman, 2004). If there is a change in the signal design, reproductive isolation may occur due to a failure to communicate if such signals are important for mate choice or species recognition (Leal and Fleishman, 2004). Variation in traits such as metabolic rates, trophic structures, acclimatisation abilities, thermal and osmotic tolerances and life history parameters can also be expressed through changes in behaviour (Ptacek and Travis, 1996).

In terms of aggression, individuals can use assessment strategies to avoid fighting individuals that they are likely to lose against in order to lower the costs and risk of injury (Caldwell, 1985). Game theory predicts that individuals will compare their Resource Holding Potential (RHP), the ability of an individual to win a fight, to that of their opponents before deciding whether or not to escalate a fight (Sneddon *et al.*, 1997). The RHP of an individual can be influence by body size, age, sex (Beacham and Newman, 1987), morphology, physiology, previous aggressive experiences (Sneddon *et al*., 1997), prior residency, energy reserves, resource value and physical condition (Morrell *et al*., 2005). Asymmetries between opponents, where the RHP of each opponent differs, for example body size, can lead to the quick resolution of fights (Maynard Smith, 1974). Interactions between closely-matched individuals, however, are likely to escalate over time and increase the risk of injury to each individual (Maynard Smith, 1974). The fight will continue until the individual with the lowest cost threshold reaches that level (e.g. expended energy levels) meaning that the duration of the fight is determined by the RHP of the individual with the lower cost threshold, the eventual loser (Morrell *et al*., 2005).

In order to decrease the risk of injury incurred during combat, communication systems capable of transmitting sufficient information to the opponent would be expected to evolve if weapons used in aggressive interactions are potentially lethal (Caldwell, 1979). In *N. acuminata*, aggression potentially involves such behaviour as the jaws are used in fights to attack the opponent, which can escalate to severe aggression involving biting (Reish and Alosi, 1968). Divergence between populations may therefore be expressed in changes in behaviour, including aggression. Dunbrack and Clarke (2003) stated that the levels of aggression in pair-wise contests should be higher the greater the behavioural divergence there is between two opponents. Individuals from different populations should display divergence that is greater than that shown between two individuals from the same population. This is known as the communication failure hypothesis and can lead to the escalation of fights between individuals (Maynard Smith and Riechert, 1984). Escalated fights are more likely when cues from the initial assessment are ambiguous and individuals must use more overtly aggressive behaviours in order to determine the outcome of the fight (Maynard Smith, 1974). Communication signals which deviate from the population norm could be sufficiently ambiguous that it provokes a more aggressive response (Dunbrack and Clarke, 2003). Evolutionary modifications of communication signals may lead to divergences in population behaviour, causing higher levels of aggression or prolonged fights between populations in pair-wise contests due to a mutual ambiguity in individual assessment (Dunbrack and Clarke, 2003). Population comparisons are therefore used to gain insight into the causes of behavioural differentiation (Lahti *et al*., 2001) and the selective influence of the environment on signal expression (Lovern *et al*., 1999). Undertaking genetic and behavioural studies using populations of a species are also important as taxonomists do not take into account the different reproductive methods used by a species, the genetic diversity between populations or the behavioural differences expressed within and between populations. In the species complex comprised of *N. diversicolor*, *N. limnicola* and *N. japonica*, all three share similar morphology but each has different reproductive and developmental characteristics (Fong and Garthwaite, 1994). *N. japonica* reproduces via a mass spawning event (Smith, 1958), *N. limnicola* is a viviparous self-fertilising hermaphrodite (Baskin, 1970) and *N. diversicolor* females reproduce in burrows with males releasing sperm in front of the female tube (Bartels-Hardege and Zeeck, 1990).

Previous studies have shown behavioural divergences within populations (Saito, 1995; Lahti *et al*., 2001). Aggression levels within populations of the brown trout *Salmo trutta* show high variation with migratory populations exhibiting higher aggression levels when compared to the aggression observed within the other populations (Lahti *et al*., 2001). Aggression has also been examined in staged contests between individuals from different populations by Dunbrack and Clarke (2003). In their study, two populations from different riverine systems of the brook trout *Salvelinus fontialis* were compared in size-matched contests, using nipping rate as the index of aggressiveness. The mean number of nips displayed between individuals from different populations was found to be greater than the mean number of nips in fights involving individuals from the same population, showing escalated fights between individuals from different populations (Dunbrack and Clarke, 2003).

Studies on the different populations started with examining the chromosome numbers of the different populations. The Reish (R) population, collected in 1964 from Los Angeles Harbour, was found to have a diploid chromosome number of 18 (Pesch and Pesch, 1980). The Newport (N) and San Gabriel (SG) populations were also found to have diploid chromosome number of 18 (Weinberg *et al*., 1990). The Newport population was collected from Newport Beach in 2005 and the San Gabriel populations collected from the San Gabriel River in 2003. The Connecticut (C) population, collected from Alewife Cove in 2002, has a diploid chromosome number of 22 (Weinberg *et al*., 1990). Differences in chromosome number can be used as indicators of reproductive compatibility (Knowlton, 1993). To determine if these populations were reproductively isolated, pre- and post-mating isolation trials have been undertaken.

Weinberg *et al.* (1990) looked at the pre-mating isolation levels between two populations from the Atlantic Ocean: Massachusetts (M) and Connecticut (C), and two from the Pacific Ocean: San Gabriel (SG) and Newport (N) and found evidence of significantly higher levels of reproductive isolation between populations. However, between the SG and the N populations, there was no evidence of pre-mating isolation. The trials were then extended by Weinberg *et al.* (1992), using the SG, N and R populations. High levels of pre-mating isolation were observed between R and both SG and N, however there were low levels of pre-mating isolation between SG and N. It was therefore concluded that speciation occurred in the R population in the laboratory following a founder event. This work by Weinberg *et al*., (1992) caused controversy as it suggested that speciation of the R population had occurred in the laboratory. The findings were therefore further discussed in the work by Rodriquez-Trelles *et al*. (1996). This work disagreed with Weinberg *et al*. (1992) and hypothesised that the two wild populations used in the study (SG and N) were not representative of the population from which the R population was originally sampled from. The study went on to investigate the genetic markers, genetic variability and divergence of the three populations, finding high genetic differences and the R population sharing no common alleles with either the SG or the N populations in 13/18 loci. The study therefore concludes that these three populations formed separate species before the R population was sampled in 1964 (Rodriquez-Trelles *et al*., 1996).

In support of the pre-mating isolation findings, D.H. Lunt and J.D. Hardege (unpublished) examined the nuclear and mitochondrial DNA from these populations. According to the nuclear DNA, the C population is likely to have diverged from the other three populations approximately ten million years ago. Examining the mitochondrial DNA, following the divergence of the C population, the SG population diverged from the R and N populations approximately seven million years ago. R and N have been found, using the mitochondrial DNA, to be very similar genetically, with little divergence.

In this thesis, experiments were undertaken using the four laboratory populations R, N SG and C, looking at the aggression between individuals over fifteen minute trials. Also, aggression and pairing behaviour was observed between the R population, sampled in 1964, and the wild Los Angeles (LA) population, sampled in 2008 from the same location as the R population. This was performed to determine if there was any divergence in behaviour within the populations which would affect the interactions of individuals between populations. It was expected that individuals from different populations of *N. acuminata* would exhibit higher aggression levels towards each other than individuals from the same population, with both males and females. As the populations all share the same life history traits and are very similar morphologically (Weinberg *et al.*, 1990), the divergence between the populations may be expressed via heightened aggression due to the communication failure hypothesis, indicating that escalated aggression would be expected between populations that have diverged (Dunbrack and Clarke, 2003). In addition, the new wild Los Angeles (LA) population (sampled in 2008) was compared to the existing laboratory maintained Reish (R) population with both males and females to determine if there are any differences in aggression between these two populations and whether these results suggest that these two populations should be treated as potentially separate species. As the R population and the wild LA populations were collected from the same geographical location, the LA population may provide a better representation of the population that R was originally sampled from. It was hypothesised, due to changes in the environmental conditions in Los Angeles Harbour due to the pollution abatement programme (Reish *et al.*, 1980) and as the R population has been maintained in the laboratory under constant light, temperature and feeding regimes, that these two populations will have diverged in the 44 years that they have been separated and that aggression levels would be higher between these two populations than within them. Aggression was found to be a weak indicator of differences between the populations, although significant differences were observed in male aggression between the populations, this was not observed with female aggression. There were significant differences in female aggression between the R and LA populations, but this was not the case for male aggression. There may be problems with using individuals in staged fights not only because this is a forced situation between two individuals, but variable may not have been taken out when comparing individuals. It is possible that individuals used in some staged contests within populations were more related than individuals used in other replicates. In many species, individuals have been shown to be less aggressive towards kin as observed in the spider mite *Schizotetranychus miscanthi* (Saito, 1995). Studies of this kind in the future should therefore take into account the kin relationships of the individuals used and avoid staged contests between closely-related individuals.

Staged contests between individuals may not have been long enough to gain a true picture of the interactions between individuals, and this may hold true for all aggressive interactions observed in this thesis. It may also be the case that the fight duration is a more important indicator or aggression than intensity of fighting. Staged contests between size-matched individuals may also have an effect on the results. As previously mentioned, symmetries in RHP can lead to escalated fights (Maynard Smith, 1974). It is possible that the symmetry in body size used in these experiments with matching opponents has led to individuals exhibiting a greater level of aggression towards each other. It is also possible that although worms were size-matched to their opponents, there were differences in the size ranges of individuals between the replicates, with some individual fights involving smaller individuals than other fights. Morrell *et al*. (2005) found that in the fiddler crab *Uca mjoebegi*, fight duration increased with increases in the mean size of closely-matched opponents. This suggests that larger individuals are capable of fighting for longer periods than smaller opponents (Morrell *et al*., 2005). In future studies, these variables should be investigating, looking at how long individuals spend fighting, observing behaviour over longer periods of time, and taking care when selecting individuals to use in staged contests, using digital equipment to document all behaviours exhibited.

Pairing observations between male and female individuals from the different populations were used to determine if there was any pre-mating isolation between these populations. Due to female death following egg shedding, mate recognition by females should be important in the species as incorrect mate recognition could lead to a wastage of gametes if eggs are shed with an ‘incorrect’ male (Snell, 1989). Before engaging in courtship, individuals need to ascertain that the potential mate is of the correct gender, of the same species, is sexually mature and receptive (Gompel and Prud’homme, 2009). Population divergence may potentially cause a divergence in sex pheromones, causing a breakdown in mate recognition (Weinberg *et al*., 1990). It was hypothesised that there would be significant pre-mating isolation between these populations when compared to pairing behaviour observed within populations. Pre-mating bioassays were also carried out between the wild LA population and the laboratory reared R population. As mentioned above, these two populations were collected from the same geographical location so the LA populations may provide a better representation of the populations that R was originally sampled from. Any differences in pair formation behaviour between these two populations may further aid to decipher the differences between the populations of *N. acuminata* currently maintained in the laboratory and it was expected that these populations will have diverged and will show significant levels of pre-mating isolation. If pre-mating isolation does occur between the R and LA populations, this would add further evidence in support of the theory that the R population has speciated in the laboratory (Weinberg *et al.*, 1990). Although the populations from the west coast of the USA are unlikely to encounter individuals from the east coast, it is still important to observe the behavioural interactions between the populations to attempt to understand the similarities and differences between these populations and to untangle any confusion regarding speciation between these populations. Pairing behaviour was observed to be a much more reliable indicator of population differences and divergence, with aggression observed between males and females in some of the replicates. There were significant differences in the pairing behaviour observed within the populations than between individuals from different populations. However, some pair bonds were found between individuals that diverged approximately 10 million years ago, between the Reish population and the Connecticut population. Even though it is thought that the Reish population is heavily inbred, this population still formed pairs with members from the Los Angeles populations and vice versa. The frequency of pair formation within the R and LA populations was also found to be significantly different to that when males and females from different populations were placed together. Although there was a significant difference, the pairing frequencies were high between the R and LA populations, with 32/40 pairs formed after 24 hours. It is possible that the experimental time of 24 hours was not long enough to determine the true pre-mating pairing behaviour between these populations. Post-mating trials were not conducted to see if any of the pair formations resulted in viable offspring. If viable offspring are produced from individuals from difference populations, this would indicate that the populations are not members of different species. The genetic relatedness of these two populations needs to be examined, along with post-mating trials to see if viable offspring are produced from any pre-mating pairs.

As it is possible that *N. acuminata* forms part of a species complex, this has to be taken into account when observing behaviour and undertaking ecotoxicology testing as sibling species may react differently in response to ecological bioassays, giving varying results to standard testing conditions (Knowlton, 1993). As *N. acuminata* have been established as an indicator species for ecological testing of pollutants (Reish, 1966), it is important to know if these population groups should be treated separately to ascertain that the results obtained for such studies are valid. In the mussel *Mytilus edulis*, the most commonly used species for biomonitoring of water quality, *M. edulis* and *M. trossulus* were collected from the same site in Canada and the body tissue analysed for twenty five different element concentrations (ppm dry weight), including Na, Zn, Cu, Ag, As, Ca, K, Al, Mg and Pb, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Even though these two species were sampled from the same geographical area, due to the differences in metabolic rate between *M. edulis* and *M. trossulus*, the concentrations of the elements tested were found to vary (Lobel *et al*., 1990). In *N. acuminata* therefore, the results of any ecotoxicology testing should be re-assessed to ascertain that effects are due to the environment and not due to any possible differences between the populations used. Further work needs to be undertaken to resolve the confusion between the different pseudonyms used for this species, including further sampling of natural populations, both pre- and post-mating trials and molecular similarities and differences. The more we know about the behavioural processes in *N. acuminata*, the more we can apply such knowledge to use this species as an alternative indicator species for pollution and water quality monitoring. This species can also be used to determine the effects of habitat change on population divergence.

Sexual selection theory predicts that mate choice will evolve when mating partners vary in genetic quality or with the ability to provide benefits to mating partners (Andersson, 1994). Direct benefits can include parental care quality, increased fertility (Searcy, 1982), provision of breeding resources (Pampoulie *et al*., 2004), nuptial gifts, absence of parasites (Møller and Thornhill, 1998), food for mates and/or predator defence (Yasui, 2001). Indirect benefits to mates can include health and genetic quality (Yasui, 2001). It is likely that the sex with the lowest potential reproductive rate should be choosy (Kraak and Bakker, 1998). Added to this, a skewed Operational Sex Ratio (OSR), the ratio of males to females in a population, can lead to increased competition between the more abundant gender (Emlen and Oring, 1977). Generally, females are choosy and males are more active in courtship, known as the ‘traditional’ male sex-role (Berglund *et al*., 1986). Reproduction in females is considered to be more costly due to the limitations of egg production and the lower reproductive rate (Pitnick and Markow, 1994). Female choice and assessment of mates has therefore led to the evolution of multiple male traits and the preference of females for such traits (Kodric-Brown, 1995). Such traits can signal to the female that males are in good physical condition and are of good genetic quality (Andersson, 1982). Traits can include superior fighting ability and/or the quality of parental care (Pampoulie *et al*., 2004). Sex-role reversal, where males are choosy and females compete to mate, is expected to occur if mating is costly in males (Amundsen *et al*., 1997), if female quality varies, if there is a female-biased OSR (Côte and Hunte, 1989), if females can re-mate faster than males (Berglund *et al*., 1992), if males have a lower reproductive rate (Svensson, 1988) or if males invest more in gametes than females (Gwynne, 1981). In many sex-role reversed species, the male is the sole provider of parental care (Ridley, 1978) and male mate choice correlates to the higher reproductive input of the male into his offspring (Berglund *et al*., 1986).

Although male *N. acuminata* undertake sole parental care (Reish, 1957), this does not necessarily mean the species is sex-role reversed (Vincent *et al*., 1992). Even though there are high levels of investment in offspring by both males and females of this species (Starczak, 1984), female reproduction costs due to female death following egg dehiscence are likely to be higher than costs incurred by males. Males are also able to reproduce again following completion of egg care (Reish, 1957), meaning males have a lower investment than females (Starczak, 1984). Also, mating is non-random (Starczak, 1984) as female choice for males has been observed and studies indicate that choice is based on chemical cues received by the female (Starczak, 1984; Fletcher, 2004; Storey, 2006). Females of this species do exhibit a preference for dominant males but overall prefer experienced males, those that have previously produced a viable brood (Fletcher 2004; Storey, 2006, Fletcher *et al*., 2009). It is therefore unlikely that this species exhibits male mate choice and there is evidence that female *N. acuminata* exhibit mate choice based on the good parent model of sexual selection, where males display to females the desired trait of completion of egg care and the successful rearing of a brood (Hoelzer, 1989).

This thesis attempted to expand upon the knowledge of female mate choice in *N. acuminata*. These can be loosely divided into female aggression, male aggression and male parental experience (including cannibalism). The experiments undertaken are all related to the unique life cycle in *N. acuminata*, and the behaviours investigated in terms of their relationship to the life cycle are demonstrated in Figure 8.1 on the next page.

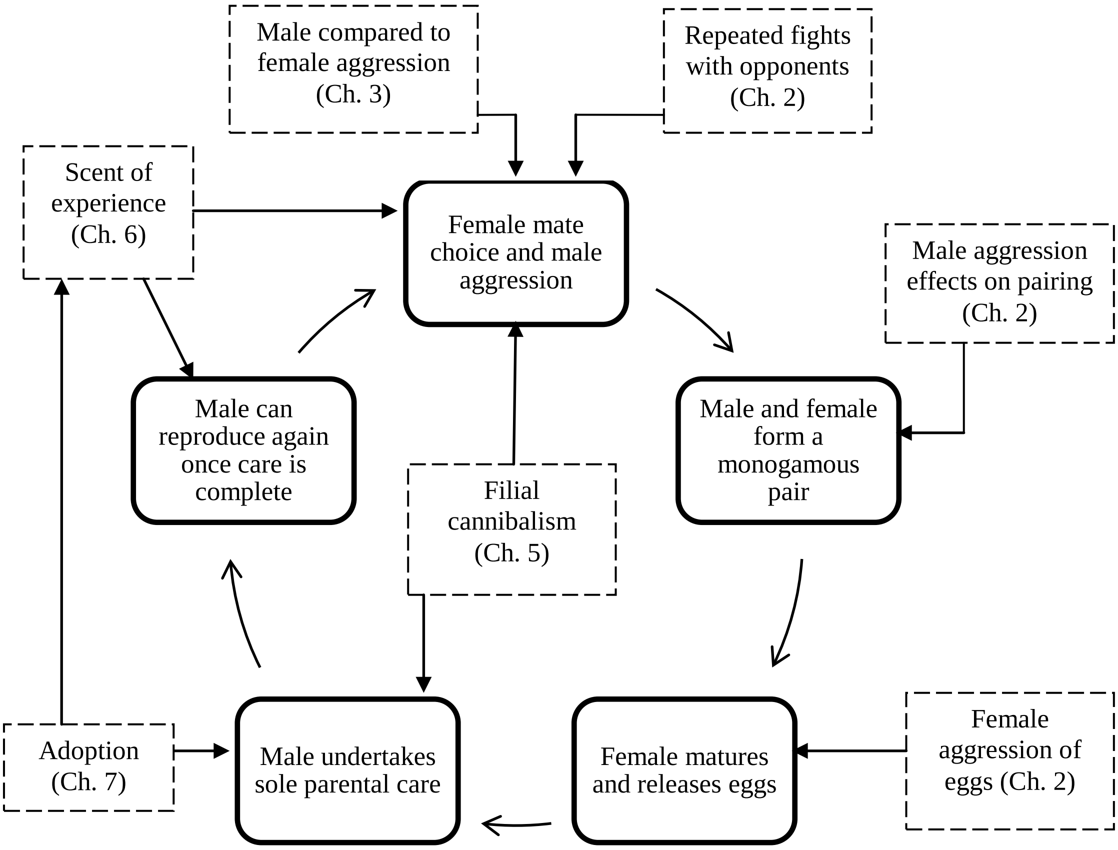


Figure 8.1: The life cycle of *N. acuminata* (bold boxes) with the different interactions observed in each chapter and their relationship to the life cycle (dotted boxes).

In nature, the male is generally the more aggressive sex due to its relationship with mating and mating success, where females are often choosy and males actively court such females, undertaking 'traditional' sex-roles (Berglund *et al*., 1986). In the case of the experimental species *N. acuminata*, Starczak (1984) observed that winning fights when an individual of the opposite sex was present was more prevalent when two males were placed with a female compared to two females placed with a male. Males are also typically the more aggressive sex if there is limited availability of females (Kvarnemo *et al*., 1995). Due to female death following egg release in *N. acuminata* and because more monogamous breeding events occur over time, it is likely that the sex ratio in a population will become male-biased (Starczak, 1984). Males are also able to reproduce again once the eggs have hatched and larvae have left the parental tube (Reish, 1957). Levels of aggression found between male individuals were compared to those found between females. As female choice and male aggression for females is evident in this species (Starczak, 1984; Fletcher *et* al., 2009), it was hypothesised that female aggression intensity will be lower than that of males. Therefore, levels of aggression were compared to determine the sex displaying the highest levels of aggression to see if this provided further indication of the occurrence of female choice in *N. acuminata*. However, there were no significant differences found in the levels of aggression displayed by males to those displayed by females. As previously mentioned, the similarity in aggression displayed by males and females could be due to the potential problems with using staged contests between individuals. Aggression may also be lower in males if the resource is not present, in this case a female, as individuals should avoid costly fighting if it is unnecessary (Caldwell, 1985).

One of the broadest areas of research concerning female mate choice has been regarding dominance hierarchies in species with male to male contests. Females select for dominance in many species (Searcy, 1982) as dominant males may possess a greater ability to provide breeding resources such as territory and female protection (Moore *et al*., 2003) and dominance may also be a good indication of reproductive success in males (Searcy, 1982). Dominant males are likely to be larger in size and this may indicate that they are superior at fighting for access to mates (Bisazza *et al.*, 1989) or that they are better able to provide protection against predators (Takahashi and Kohda, 2001). Females have also been found to “eavesdrop” on aggressive contests between males to gain information regarding mate quality, as observed in the female Siamese fighting fish *Betta splendens* (Doutrelant and McGregor, 2000). This ensures that females mate with high-quality males (Forsgren, 1997) as success in contests is indicative of good male condition (López *et al*., 2002). Previous studies have examined the dominance relationships in *N. acuminata* (for example Starczak, 1984; Fletcher *et al*., 2009) but these studies do not appear to have taken into account whether the aggressive bouts themselves have any subsequent effect on pairing behaviour when males are placed with females. The outcome of aggressive interactions may influence the ability of each male to attract a mate and may affect female choice (Kangas and Lindström, 2001). This could therefore have an impact on results obtained in studies looking at the aggressive interactions between males, for example if males are returned to fight with one another. The effects of an aggressive bout on the subsequent time it takes for males to form a pair bond with a female, seen when males and females lie alongside each other ‘head to tail’ and start to construct a new mucous tube were investigated in *N. acuminata*.

In some species, although male to male contests and the formation of dominance hierarchies can be observed, females do not always select for the dominant individual (Moore *et al*., 2003). In species with male parental care, the ability to raise offspring may be the deciding factor in mate choice, with good fathers preferred (Wong, 2004). Hatching success must be heavily reliant on male care for females to select mates based on their parental care ability and therefore, the selected trait conferring the signal to the female must be reliable (Hoelzer, 1989). Dominance may not provide such a reliable indication of parental care ability (López *et al*., 2002) and dominant males do not always make better fathers (Wong, 2004). Hatching success in the sand goby *Pomatoschistus minutus* has been shown to be dependent on sole paternal care and males with nests containing eggs are preferred over those with empty nests (Forsgren *et al*., 1996). Added to this, dominant males were not found to be better at egg care and females gained no direct benefits from mating with such males (Forsgren, 1997). In *N. acuminata*, females have previously been shown to select for males experienced in parental care, rather than males showing overt aggression (dominant males) (Fletcher *et al*., 2009). It was therefore expected that, as male parental care is vital in this species due to female death following reproduction (Reish, 1957), pairing with males capable of performing such care would be more important to females than male fighting ability and the occurrence of an aggressive interactions would increase the time taken for a male and a female to pair. The results showed that there was no such decrease in aggression. Males and females that were familiar with each other did not show significant reductions in pair times. It is therefore possible that aggression does not influence pairing behaviour with a female in *N. acuminata* and that there is no recognition for individuals that have previously formed a pair bond.

Dominance hierarchies can be maintained when individuals that have previously fought are able to recognise each other (Gherardi and Teidemann, 2004). Recognition occurs when each individual in a group can be discriminated from every other individual based on a unique set of cues that define that individual (Karavanich and Atema, 1998). Individual recognition has been demonstrated in the hermit crab *Pagurus longicarpus*; individuals were more aggressive during fights when placed with unfamiliar opponents (Gherardi and Teidemann, 2004). Recognition of previous opponents has also been shown in male *Homarus americanus* lobsters, where previous encounters are remembered and the subsequent aggressive response is lowered (Karavanich and Atema, 1998).

The outcome of prior aggressive interactions can influence an individual's behaviour in subsequent interactions. Winning or losing fights can increase or decrease various measures of fighting motivation and affect the ability of the individual to win in a subsequent encounter. Winners of fights may keep winning, and losers keep losing (Collias, 1943 cited Jackson, 1991). In the pumpkinseed sunfish *Lepomis gibbosus*, prior winners of a fight defeated unfamiliar prior losers (Beacham and Newman, 1987). As escalated or prolonged fight pose a considerable risk to both opponents, recognition of former opponents may also reduce this risk of injury and reduce the costs associated with aggression (Caldwell, 1985). Subsequent aggressive interactions between individuals were observed between males to determine if there was any recognition of opponents from previous fights. There was no observed decrease in the intensity of fighting between individuals. However, as mentioned above, the intensity of aggressive displays between individuals may not be important, instead fight duration may provide a better indication of aggressive behaviour. Learning is reported to be slow and short-lived in polychaetes. It is possible that fighting individuals for fifteen minutes and then separating them for two hours does not lead to recognition of previous opponents, because the fight duration was too short, because the separation period was too long, or both.

All these interactions potentially affect the formation of a pair bond between a male and a female. After individuals have formed this pair bond, females release their eggs to be fertilised and cared for by the male. Females are expected to be more aggressive towards another female intruding into her mate's territory/showing interest towards her mate, according to the investment guarding hypothesis (Yasukawa and Searcy, 1982). When one sex makes substantial contributions of parental investment, sexual selection can favour the evolution of adaptations that protect the investment (Trivers, 1972 cited Yasakawa and Searcy, 1972). Aggression in females can be heightened during the breeding season (Slagsvold, 1993) and intruders to the nest area may affect the resident female in a number of ways. Females that settle nearby with a mate can increase the competition for resources, but intruding females may also destroy offspring, parasitize the nest, displace the resident female or her mate and/or settle with the resident female's male, therefore decreasing the paternal care extended to her offspring (Slagsvold, 1993). Such aggression from females has been suggested to be important in maintaining the monogamous relationship between males and females (Wittenberger and Tilson, 1980) and also secures full male assistance with caring for offspring (Davies, 1989 cited

Sandell, 1998). The aggressive behaviour of individuals has also been shown to change when offspring are present and over the course of the breeding season. In the scissortail sergeant *Abudefduf sexasciatus*, males care for eggs solely and are only aggressive during this period (Manica, 2010). Several studies have shown that whilst in the presence of offspring, females also increase their aggression levels in order to protect the reproductive investment, known as maternal aggression (Tallamy and Denno, 1981). Such aggression has been observed in the white tailed ptarmigan *Lagopus laucurus*, with females observed to exhibit heightened aggression towards other conspecific females whilst caring for eggs (Martin *et al*., 1990). Individuals caring for offspring also increase the intensity of aggression as the offspring get older and the probability of survival increases (Jaroensutasinee and Jaroensutasinee, 2003).

In *N. acuminata*, a spent female has been observed to fight a sexually mature female if one is introduced to the dish that houses the male caring for her eggs (Reish, 1957), presumably to prevent the female from attempting to mate with the male guarding her eggs, therefore protecting her reproductive investment. As females of this species die following reproduction, the survival of eggs depends entirely on the male and females should protect their reproductive investment, not only by selecting a good quality male to care for her eggs, but also by preventing the male from reproducing with another female and deserting or cannibalising the spent female's eggs. This thesis examined the aggressive response of females that had laid eggs to sexually mature females when spent females were removed from the vicinity of their egg mass and the male caring for them. It was expected that the aggression levels shown by spent females would be less than that between sexually mature females as the female is no longer in the presence of the egg mass. No aggression was observed between spent and sexually mature females. Dehiscence causes a breakdown in the body wall and changes in the musculature (Starczak, 1984) which may have affected fighting ability. The spent female was also no longer in the presence of her egg mass so there would be no benefit to displaying aggressive behaviour.

Female choice for males with parental experience has been demonstrated in *N. acuminata* (Fletcher *et al*., 2009) and this thesis examined this occurrence in more detail, looking at filial cannibalism and female preferences, conditioned water experiments and also adoption of eggs fertilised by another male.

Cannibalism of eggs by a parent, termed filial cannibalism, may occur for a number of reasons. Aside from accidental consumption (Schabetsberger *et al.*, 1999), cannibalism may serve to clean nests of dead or diseased eggs (Kraak, 1996) but it also provides the parent with nutrients and energy (Polis, 1981). Parental care may be costly in terms of reduced foraging opportunities (Lindström, 1998) so filial cannibalism may also function to offset the cost of such care (Okuda and Yanagisawa, 1996). Consuming eggs may not fully compensate for the cost of care (Kraak, 1996) but has been found to have the potential to increase future reproductive success by survivorship of the parent (Lindström and Sargent, 1997). Energy can therefore be redirected towards attracting new mates or defending a nest site for longer (Peterson, 1990). In the cardinal fish *Apognon doederleini*, Okuda and Yanagisawa (1996) found that males were more likely to cannibalise eggs if they were expecting to re-mate quickly. When mate availability is high, males can easily obtain matings from additional females (Kondoh and Okuda, 2002). Filial cannibalism may also occur when the brood is smaller than normal as the reproductive value of the clutch would not outweigh the costs associated with care of that brood (Petersen and Marchetti, 1989).

Filial cannibalism is more prevalent in species with male parental care (Okuda *et al.*, 1997) due to the large gametic investment made by the female compared to the smaller parental investment made by the male (FitzGerald, 1992). To counteract losses from predation and cannibalism, females have been observed ‘copying’ the mate choice of previous females by laying eggs in nests already containing egg masses (Rohwer, 1978). Such behaviour has been observed in teleost fish and increases the chances of a female selecting a good quality male. Copying also increases the level of care to the brood due to its larger size and dilutes the predation risk (Kraak, 1996). Females have also been observed using test eggs, small clutches containing only a few eggs, to test the parental abilities of males (Kraak and van den Berghe, 1992; Manica, 2010). Males were significantly less likely to receive additional eggs if the small clutches were not present after one day, compared to those received when the small clutches remained (Kraak and van den Berghe, 1992). Females therefore test the quality of male care by laying such clutches and males should therefore be prepared to care for small numbers of eggs in order to increase their chances of receiving additional matings (Kraak and van den Berghe, 1992). However, few studies have gone into detail regarding sexual selection by females for or against cannibalistic males.

In the experimental species *N. acuminata*, filial cannibalism has been observed with males caring for offspring (Oshida *et al*., 1981) with both experienced and inexperienced males consuming eggs (personal observation). Female *N. acuminata* make a larger gametic investment than males due to female death following reproduction (Starczak, 1984) whereas males make a larger parental care investment than females (Reish, 1957). Females should select good quality males (Hoelzer, 1989) and filial cannibalism may be a way for males to increase their physical condition prior to undertaking future parental care (Klug and St. Mary, 2005) and this may serve as an advantage to *N. acuminata* females looking for a male able to undertake sole parental care once the female has laid her eggs and died. Males in good physical condition are less likely to need to cannibalise eggs during egg care (Manica, 2010) and females should minimise the chances of the reproductive investment being lost due to the eggs laid becoming food (Petersen, 1990). The occurrence of male filial cannibalism can therefore exert a selective influence on female reproductive tactics (Petersen, 1990).

Female preferences were examined in *N.* acuminata to determine if a female can detect that a male has cannibalised an egg mass under his care and if so, whether this influences female choice of male, with females preferring to mate with either an inexperienced or an experienced male over a cannibalistic male. As sole male parental care occurs in this species (Reish, 1957) and females make a larger gametic investment than males (Starczak, 1984), the female should select a male in good physical condition, capable of rearing her brood to avoid her reproductive investment being wasted (Hoelzer, 1989). It was expected that females would not avoid pairing with a male that had previously cannibalised an egg mass. However, the results were found to be confusing as female choice was equal when given a choice between cannibalistic males and inexperienced males, but also equal when give a choice between cannibalistic males and experienced males. Females should want to select the best male possible to care for her eggs (Holezer, 1989) so it is possible that cannibalism is an intermediate preference for females, preferred over inexperienced males but not preferred over experienced males. More replicates need to be carried out to determine the extent of female preferences in relation to cannibalism in *N. acuminata*.

Once males have reared a brood, they become experienced in parental care and are preferred by females over inexperienced males (Fletcher *et al*., 2009), even though there are high levels of reproductive investment made by both males and females (Starczak, 1984). There is also evidence that choice is based on chemical cues received by the female (Starczak, 1984; Fletcher, 2004; Storey, 2006). Although these findings add support to the evidence that female choice occurs in this species, it is still not entirely clear at which point the male becomes preferred by a female, either by a morphological, behavioural or hormonal change in the male, and if such a preference is signalled to the female chemically.

Conditioned water bioassays have been used to determine if water-borne chemicals are used for communication both between and within species, using surrounding water taken from certain individuals that can then be used in choice trials to determine the effect (if any) of chemical signals that may be present in the water sample. Conditioned water has been used to examine the behavioural responses in *N. acuminata*. Brown (2005) found that aggression was lowered between males originating from different populations when exposed to conditioned water from the opposing population. It was also found that female choice changed in *N. acuminata* when males were placed in conditioned water originating from males undertaking parental care of an egg mass. Previous losers of female choice were subsequently chosen by the female after they were placed in conditioned water from parental males (Fletcher *et al*., 2009).

Observations were made of both male and female behaviour following the masking of a male with conditioned water from various sources. Male aggressive behaviour was observed following masking. As previously mentioned, individuals use assessment strategies to determine the RHP of their opponent and the likelihood of winning a fight (Maynard Smith, 1974). Placing males in conditioned water from females, juveniles and inexperienced males was not expected to alter the aggressive behaviour observed between individuals, as the RHP of the masked opponent (an inexperienced male) should not be altered by the conditioned water. However, conditioned water from males caring for eggs and both new and old experienced males was expected to alter the aggression observed between two opponents as the RHP of the masked male would have changed due to the ‘presence’ of a male undertaking egg care or an experienced male. Female choice behaviour was observed to determine if female preference was artificially altered following the use of such conditioned water and if a water-borne chemical cue is involved in female choice and reproduction in *N. acuminata*. Experienced males convey chemically to females that they have previously undertaken egg care, as previously shown with conditioned water from males caring for eggs

(Fletcher *et al.*, 2009) but it is unknown if the same effect will be observed with conditioned water from other sources. Individuals in different stages of the life cycle of *N. acuminata* were used to condition water and these include juveniles, sexually mature females, inexperienced males, males undertaking egg care, new experienced males and older experienced males. Conditioned water from older males was also used to observe if there was an effect on female choice due to the age of the male. As females have been shown to prefer experienced males over inexperienced males, it was expected that males placed in conditioned water from new and old experienced males would alter subsequent female choice, as would the conditioned water from males caring for eggs. It was expected that the conditioned water from females, juveniles and inexperienced males would not alter subsequent female choice as these should not alter the initial choice of mate made by the female. It was found that male aggression was not altered following the use of conditioned water in all treatments except when using conditioned water from old experienced males. Age-related dominance has been observed (Hollister-Smith *et al.*, 2007) so it is possible that older males are more likely to win fights between individuals and this was conveyed in the conditioned water that the inexperienced male was placed in. A size difference may also be conveyed in the conditioned water, leading to a quick resolution of fights due to asymmetries between opponents (Maynard Smith, 1974). Conditioned water did have an effect on the subsequent choice made by the female. With the trials involving water from new experienced males and males caring for eggs, female choice switched from a previous winner to the masked loser. It is possible that a change occurs during egg care that confers the parental care ability of the male, causing the female to prefer the masked female over the other inexperienced male. In the common marmoset *Callithrix jacchus*, levels of prolactin have been found to vary between males that have not undertaken care of offspring and those that have successfully reared a brood (Schradin and Anzenberger, 2004). In the trials involving water from females, juveniles and inexperienced males, female selected the previous winner in significantly more cases than in the trial using sea water. In these trials, the use of conditioned water appeared to reinforce the original choice made by the female. However, these types of trials only present the female with two males, 'forcing' the female to choose between the two males in order to complete the experiment. The female should avoid wasting her lifetime's reproductive effort (Booksmythe *et al.*, 2008) so future studies should be undertaken using 'free-mixing' experiments, if females are given a choice of multiple males rather than just two, to help to understand the processes involved in female choice in *N. acuminata*.

Related to female preferences for experienced males, adoption was examined in *N. acuminata*. Adoption is defined as the care of offspring by individuals that are not the genetic parents (Wilson, 1975 cited Riedman, 1982) and has been reported in fish, bird and mammal species (Riedman, 1982). Adoption is hypothesised to be reproductively costly and inconsistent with classic evolutionary theory (Riedman and Le Boeuf, 1982) but the benefits of adoption may offset such costs incurred (Brown, 1998). During adoption, parental behaviours may be transferred to offspring through imprinting and may include preferences for food, mate choice, a particular niche preference or foraging techniques (Avital *et al*., 1998).

Adoption may form a true altruistic act with no selfish benefits to the carer, as observed in social insects such as termites, wasps, bees and ants where sterile castes exist that care for offspring of the queen (Wilson, 1975). However, such unselfish behaviour is rare and it is more likely that the carer will receive benefits in some way, making the act selfish. Such benefits may include receiving an increased number of matings due to caring for offspring, enjoying increased social status, increased access to resources such as food or shelter, gaining parental experience and enhancing parenting skills (see Daniels, 1979 for review). Caring for young may influence the future reproductive success of the male (Riedman and Le Boeuf, 1982) and may be favoured by natural selection to enhance the adoptive individuals parenting skills (Riedman and Le Boeuf, 1982). This is especially important for species where competent parenting skills are vital for example if females produce a very limited number of eggs (Riedman, 1982). Tallamy (2000) postulates that if the presence of an egg mass increases the attractiveness of the male, unrelated eggs should be accepted by the male but also competed for against other males. Therefore, males should be willing to care for unrelated eggs if such care attracts additional mates and therefore increases reproductive success (Tallamy. 2000). Caring for related offspring may be a form of kin selection (Daniels, 1979), aiding a closely related individual and therefore influencing its own genetic fitness (Riedman, 1982). Individuals may also assume parental care roles if it is an act of reciprocity; if the favour is returned later, for example where alloparents receive help with their offspring in turn (Daniels, 1979). In the cichlid *Tilapia rendalli*, four parents care for two brood sets which may lower the risk of predation to both sets of offspring (Ribbink *et al*., 1981). In harsh or stressful environments where breeding resources such as food and space may be limited, parental cooperation may be beneficial to both parties (Riedman, 1982). The act of adoption may occur as a result of a mistake.  Mistaken identity and subsequent brood mixing may occur if parents are unable to recognise their own offspring (Daniels, 1979; Silk, 1999).  However, parental care may also be forced upon the individual.  If parents cannot recognise their own offspring, other parents may place offspring with such individuals so as to avoid parental care duties themselves, known as brood parasitism or cuckoldry, where eggs are laid in the nests of other individuals (Weller, 1959).  The classic example of cuckoldry is found in the cuckoo *Cuculus canorus* where females lays eggs in a host's nest, such as the meadow pipit *Anthis pratensis*, and removes one or more of the host species' eggs (Lack, 1963).  Such parasitism benefits the genetic parents as the offspring will carry their genetic material, but the parents do not incur the costs associated with parental care (Wisenden and Keenleyside, 1992).  However, in some species, this may still provide a benefit to the adoptive parent as an increased number of offspring equals a dilution of predation risk (Smith, 1968 cited McKaye *et al.*, 1992).  Not only does egg survival increase with the number of eggs present, but foreign young accepted into an existing fry group may aid differential predation so smaller young may be accepted that are more likely to be preyed upon (Smith, 1968 cited McKaye *et al.*, 1992).  Kidnapping of broods also occurs, for example in the cichlid *Cichlasoma citrinellum* and it is hypothesised in this species that this dilutes the predation risk for the individual's own brood (McKaye and McKaye, 1977).

Due to parental death or abandonment, *N. acuminata* eggs normally under parental care may be found uncared for in natural populations, although this is rare as males caring for eggs hardly leave the egg mass, except to forage at the mouth of the burrow (Reish, 1957).  Approximately 10-15% of males abandon the parental tube containing developing eggs (Reish, personal communication).  Eggs left in a tube will not survive longer than two days (Reish, 1980a) and will turn a white colour when dead (personal observations).  A preliminary study by Sutton (1998) found that males would care for eggs of another male after parental males have been removed from the burrow and replaced.  Replacement males will undertake parental care following the paternal male death and hypothesised that inexperienced males would undertake such care in order to gain 'experience' and subsequently be preferred by females in future reproductive events (Reish, personal communication).  As females prefer experienced males (Fletcher *et al.*, 2009), females may select mates on the basis of their parental care abilities (Holezer, 1989).  The completion of egg care also signals to the female that the male is likely to be of high quality as he has survived to produce offspring despite the high costs associated with egg care (Tallamy, 2000).

Adoption was examined to determine if care of eggs from another male occurs under experimental conditions in the laboratory and if there was a difference in the behaviour of experienced males (those that have previously undertaken egg care) and inexperienced males. It is hypothesised that care of eggs fertilised by another inexperienced male occurs under experimental conditions and also that experienced males will not care for eggs that they have not fertilised. It was hypothesised that experienced males gain no benefit from such behaviour whereas inexperienced males may benefit by increasing their parental experience. The results showed that there were significant differences in male behaviour between the different reproductive states of males. Experienced males did not undertake any form of care, instead cannibalising the eggs in some replicates. In inexperienced males however, males did undertake parental care of eggs fertilised by another male. Experienced males would not gain any benefits in terms of increased mating by looking after another male's eggs, instead gaining in physical condition by consuming them. An inexperienced male would gain in terms of increased matings from females due to increased parental care experience. It is unknown, however, whether such adoption and selection for experienced males occurs in the wild.

With all experiments, the test situation may not truly reflect natural behaviours occurring in the wild and the results may not predict the levels shown in the original environment (Giles and Huntingford, 1985). For example, females of the sand lizard *Lacerta agilis* are not aggressive in nature towards males or females but in captivity, have been observed competing over food using biting (Olsson, 1994). As *N. acuminata* individuals are very difficult to observe in natural burrows (Starczak, 1989), it is possible that part or all of the life cycle of *N. acuminata* only occurs under laboratory conditions and not in natural habitats. Even if this is the case, this species can still be used to observe the behavioural characteristics that occur at every stage in the life cycle and used as a model species to investigate the processes of sexual selection and evolution.

It is likely that this species forms a species complex and therefore, the results of any ecotoxicology testing should be re-assessed to ascertain that effects are due to the environment and not due to any possible differences between the populations used. Further work needs to be undertaken to resolve the confusion between the different pseudonyms used for this species, including further sampling of natural populations, both pre- and post-mating trials and molecular similarities and differences. The more we know about the behavioural processes in *N. acuminata*, the more we can apply such knowledge to use this species as an alternative indicator species for pollution and water quality monitoring

This thesis attempted to examine the role of aggressive relationships and male parental experience in mate choice in the polychaete *N. acuminata*. As previously mentioned, the more we know about the evolutionary and behavioural processes in the polychaete *N. acuminata*, and the interactions between the populations, the more we can apply that knowledge to undertake environmental studies, using this species as an indicator of pollution and water quality monitoring. We can also use this species as a model for evolution as it has an easily adaptable life history, a relatively short life cycle and is convenient to use for behavioural bioassays. Observations are easy to make and experiments relatively simple to conduct at each stage of the life cycle.

It is clear that this study poses more questions than it answers. The mechanisms involved in male and female recognition in *N. acuminata* are still unclear, especially in natural environments. How populations behave and respond to multiple stimuli in the wild is still unknown. Populations used in this series of experiments have all been kept in the laboratory for at least ten years (apart from the newly sampled wild Los Angeles population) and the effects of this on the populations housed in the laboratory have yet to be investigated. Notwithstanding this, it is postulated here that this species is ideal to use as a model organism to assist in unravelling the behavioural and evolutionary processes involved in mate choice and aggression, sexual selection for parental experience and the chemical signalling involved in such a species, due to its adaptable life history, relatively short life cycle and ease of maintenance in the laboratory.

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