

LARVAL RELEASE RHYTHMS AND LARVAL BEHAVIOR OF PALINURID
LOBSTERS: A COMPARATIVE STUDY

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

This dissertation investigated larval release and larval behavior of the Caribbean spiny lobster *Panulirus argus* and the spotted spiny lobster *P. guttatus*. These species were examined under laboratory conditions to determine the phase relationship between larval release and natural environmental cycles. *P. argus* displayed a nocturnal tidal rhythm, while *P. guttatus* displayed a circadian rhythm in larval release. *P. argus* releases larvae near the time of nocturnal high slack water, while *P. guttatus* released larvae near the time of sunrise.

The role of 'pumping pheromones' in controlling larval release behaviors was tested by measuring the pumping response of ovigerous *P. argus* to (1) hatch water, (2) homogenized-embryo water, (3) embryo-conditioned water, and (4) water containing homogenized-egg cases. Lobsters with late-stage embryos displayed increased pleopod pumping with increased concentration of hatch water. Water individually conditioned with homogenized late-stage embryos, intact late-stage embryos, and homogenized egg-cases induced pumping activity in females with late-stage embryos, indicating the presence of a chemical cue.

I quantified pumping responses upon exposure to synthetic peptides to determine if they mimicked pheromones that induce larval release behaviors. Pumping behavior was evoked by oligopeptides with a basic amino acid at the carboxy-terminus,

preceded by several neutral amino acids. Carboxyl-terminal arginine peptides serve as pheromone mimics.

I investigated whether these peptides originate from the action of trypsin-like enzymes by conducting a bioassay measuring pumping activity of ovigerous *P. argus* subjected to increasing concentrations of trypsin, trypsin inhibitor, and a combination of the two. Pumping activity increased with increasing concentrations of trypsin and trypsin inhibitor, while behaviors ceased when ovigerous females were subjected to a complex of the two. Pheromones are generated by trypsin-like enzymes assisting in the degradation of the egg membranes at the time of hatching.

Vertical swimming behaviors of stage-I phyllosoma larvae of *P. argus* and *P. guttatus* were observed under laboratory conditions. *P. argus* larvae displayed a pattern of twilight vertical migration, while *P. guttatus* larvae displayed nocturnal diel vertical migration (DVM). Rhythms persisted for 5-6 cycles under constant conditions, indicating that an endogenous rhythm in activity plays a proximate role in DVM for both species.

Dedication

This work is dedicated to the memories of my father, Charles A. Ziegler II, my sister Christine J. Ziegler, my aunt Susan F. Holl, and my best friends Loretta D. Abel and E. Donnon Murray. I also dedicate this work to Lonny A. Anderson, whose contributions were the most significant.

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1. Introduction

Synchronized release of larvae is common among decapod crustaceans living in estuarine and marine habitats, and patterns in hatching are typically associated with lunar, day/night, and tidal cycles (reviewed by Forward 1987). Among the Crustacea, studies of the timing of larval release relative to environmental cycles have largely focused on brachyuran crabs in which the rhythms are typically under endogenous control (DeCoursey 1983; Forward 1987; Morgan 1995). Few studies have focused on tropical species of lobsters. Although the timing of larval release has been investigated in the homarid lobsters (Branford 1978; Moller and Branford 1979) only one report documents the timing of larval release among palinurid lobsters (MacDiarmid 1985).

This dissertation examines the mechanisms involved in larval release for two sympatric species of spiny lobsters. It begins with an introduction to the family Palinurida and provides background information for the future chapters.

1.1 Background

The family Palinurida includes the spiny lobsters which are widespread and occur in most of the temperate and tropical regions of the world (Kanciruk 1980). The palinurid life history includes the release of a unique pelagic larva, a phyllosoma, after embryonic development. These larvae are transported from coastal to oceanic waters, for a larval period of 5–12 months (Phillips and Sastry 1980; Booth and Phillips 1994;

Phillips 1994; Yeung and Lee 2002; Goldstein et al. 2006). Spiny lobster larvae appear to be poor horizontal swimmers but are strong vertical swimmers.

To ensure the greatest chances of larval survival, many species of decapod crustaceans have developed larval release rhythms with respect to various environmental cycles, including lunar, tidal, and light:dark (LD) cycles (Forward 1987). Through the temporal and spatial regulation of larval release behaviors, spiny lobsters may guarantee that offspring are placed in a suitable environment at a time when they can best survive. The extent to which larvae remain near their source population may be related to the magnitude of dispersal and self-recruitment. In other words, the closer those larvae remain to the source population, the greater the probability of recruiting back to the same population.

Long-distance larval dispersal patterns can have a critical effect on population dynamics and management of spiny lobsters (Botsford et al. 1994), given the long duration of the larval period. While the capacity for long-distance dispersal exists for palinurid lobsters, it is not known if larvae found far from suitable adult habitats typically survive to recruit or whether long-distance dispersal represents the prevalent pattern among successful recruits (Phillips and McWilliam 1986; Cowen et al. 2000). Export into coastal and oceanic environments may depend on the presence and strength of cues to which larvae respond. Phyllosoma larvae may possess the ability to travel considerable distances in the pelagic realm, and must locate or be transported to a

suitable environment for proper development. The probability of returning to the natal area (area from which spawned) is likely to be low if information cannot be gained on the direction of suitable habitat and possibly the distance from it (Kingsford et al. 2002). Although oceanographic currents can enhance the dispersal of an organism, this is unlikely to be the full story. Armsworth (2000) concluded that larval behavior is much more important for dispersal than hydrodynamic considerations of incidental dispersal, or passive entrainment.

Studies of the larval transport of palinurid species are mainly speculative, since it is difficult to study the behavioral processes in the field as phyllosoma larvae are in low abundance, widely dispersed, and transparent. Hence, our knowledge of the dispersal mechanisms used by phyllosoma larvae for orientation in the pelagic environment is poor (Phillips and Sastry 1980). The studies of hatching rhythms and early larval behaviors presented in this dissertation are critical for parameterizing oceanographic transport models that are capable of predicting the spatial distributions and transport of larvae (Kingsford et al. 2002; Sponaugle et al. 2002). These results also strengthen the current conceptual models for dispersal of crustacean larvae (*e.g.*, Epifanio 1995; Garvine et al. 1997) by including members of the Palinurida.

1.2 Life History of Study Species

1.2.1 *Panulirus argus*

The Caribbean spiny lobster *Panulirus argus* (Latreille, 1804) is distributed throughout the Caribbean, the south Atlantic, and the Gulf coast of the United States, and is among the region's leading marine resources. *P. argus* is a migratory species with a complex life cycle. In Florida, peak mating activity occurs between February and April (Lewis 1951). The mating process begins with courtship displays by both male and female lasting from hours to days (Lipcius et al. 1983). The male deposits an external spermatophore on the sternal region of the female. The female scratches the spermatophore and uses the exposed, non-motile sperm to fertilize the eggs (Talbot and Summers 1978; Martin et al. 1987). Eggs are then brooded until they hatch 15 to 21 days later, depending on water temperature (Chubb 1994). Females can carry 230,000–700,000 eggs per brood, with increasing numbers of eggs with increasing female size (Lipcius and Herrnkind 1987). The color of the egg mass changes from bright yellow-orange at oviposition to dark brown color shortly before larval release. Spawning occurs throughout most of year in the Caribbean Sea, whereas seasonal spawning occurs further north in Florida and Bermuda populations (Lyons 1980; Hunt and Lyons 1986).

The phyllosoma larvae of *P. argus* are planktonic for 6–12 months in offshore waters during which they develop through approximately 11 stages (Lewis 1951). Recent laboratory rearing experiments indicate that *P. argus* larvae can develop to the

final stage phyllosoma in less than 6 months (Goldstein et al. 2006). At the end of the oceanic phase, the final stage phyllosoma metamorphoses into a puerulus.

Metamorphosis probably takes place offshore, since late-stage phyllosoma are absent from inshore waters in Florida. Along the coast of south Florida, pueruli undertake an onshore migration across the continental shelf to settle in coastal reef areas. Pueruli either arrive at settlement sites during the new moon by horizontal swimming (Calinski and Lyons 1983) or in Florida Bay they are transported shoreward at night from November to March (Marx 1986). The pueruli settle in nearshore vegetated habitats of red macroalgae *Laurencia* spp. and seagrass where they then metamorphose into the benthic juvenile stage.

P. argus juveniles appear to undergo ontogenetic shifts in habitat use and behavior. Young juveniles (1–3 months after settlement) live in vegetated habitats where they are solitary and cryptic, whereas older juveniles (referred to as the “post-algal” stage) dwell gregariously under sponges, coral heads, or in crevice shelters associated with hard-bottom communities (Marx and Herrnkind 1985; Herrnkind and Butler 1986, 1994; Childress and Herrnkind 1996). After 2–3 years, adult lobsters migrate to the outer reef for mating and spawning (Davis 1977; Davis and Dodrill 1980, 1989; Kanciruk 1980).

1.2.2 *Panulirus guttatus*

The spotted spiny lobster *Panulirus guttatus* (Latreille, 1804) is a small (15–50 mm CL), rather sedentary species that lives exclusively on coral reefs throughout Florida, the Caribbean, and Bermuda (Robertson and Butler 2003). *P. guttatus* is an important fishery species in Bermuda, the Caribbean, and Mexico (Evans and Evans 1995), with a complex life cycle similar to *P. argus*. Although co-occurring with the congeneric *P. argus*, *P. guttatus* is not found in the seagrass or sandy areas far from the coral reef.

Mating occurs year round in the Caribbean, and occurs from March to June in Florida (Robertson and Butler 2003). Courtship and mating behavior for *P. guttatus* has not been described. A spermatophore, similar to that described for *P. argus*, is deposited on the female after mating. Females spawn 3–4 times per year, depending on their size (Sharp et al. 1997), and have an egg incubation period of 2–3 weeks (Negrete-Soto et al. 2002). Females carry 27,000–190,000 eggs per brood. After hatching, phyllosoma larvae molt through 11 stages and develop in offshore areas for presumably 9–12 months. Late-stage phyllosoma larvae (stages VI–IX) are found in near-shore waters throughout the winter in Bermuda and Florida (Evans and Evans 1995; Lyons and Hunt 1997). Metamorphosis into a puerulus may take place in near shore waters, in contrast to *P. argus* (Baisre and Alfonso 1994; Evans and Evans 1995), which metamorphoses in offshore waters.

The life history of the spotted spiny lobster is distinctly different from *P. argus* in that all the benthic stages from puerulus settler to adult appear to solely occupy coral reef habitats (Sharp et al. 1997). The postlarvae of *P. guttatus* recruit directly to holes on the underside of coral heads on shallow reefs where they remain for their entire benthic existence. Juveniles do not undergo ontogenetic shifts in habitat use or behavior, but rather remain solitary, settling in cryptic den cavities created by reef-boring molluscs (Sharp et al. 1997). Moreover, individuals of *P. guttatus* are highly reclusive and only leave their shelters for short periods during darkness. The residency of this species in a single, often patchy habitat type lies in stark contrast to *P. argus* and most other well-studied spiny lobsters, which are typically nomadic and exhibit ontogenetic shifts in habitat during their life cycle (Chittleborough 1974; Herrnkind and Butler 1986; Jernakoff et al. 1994). *P. guttatus* remains in coastal waters south of the Florida Keys near small patch reefs and is rarely found in the Florida Bay area (Sharp et al. 1997).

1.3 Larval Release: Behavior, Timing, and Control

The phenomenon of synchronous release of larvae has been documented for members of the Brachyura in whom larval release coincides with lunar, diel, and/or tidal cycles (DeCoursey 1983; Forward 1987; Morgan 1995). For species that display a diel rhythm, larval release typically occurs during darkness. However, several species of crabs (e.g., *Callinectes sapidus*, *Neopanope sayi*, *Uca beebei* and *U. lutea*) release larvae during the day or night (De Vries and Forward 1991a, b; Morgan and Christy 1995;

Yamaguchi 2001; Ziegler 2002). Yet, for species in which larval release is associated with the tidal cycle, release generally takes place around the time of high tide (reviewed by DeCoursey 1983; Forward 1987; Morgan 1995). This rule applies equally to crabs from subtidal, intertidal, and supratidal habitats, whether they are coastal or estuarine (Forward 1987). Larval release appears to be under endogenous control since animals placed under constant laboratory conditions continue to release larvae near the time of expected high tide or darkness (Ennis 1973; Branford 1978; Moller and Branford 1979; Bergin 1981; Forward et al. 1982; DeVries and Forward 1989).

Larval release has been studied for a few lobster species, but not in great detail. For the homarid (clawed) lobsters *Homarus gammarus*, *H. americanus*, and *Nephrops norvegicus* hatching occurs after sunset with the hatching process lasting for about 1 min when ovigerous females are held under a natural illumination cycle. A female releases larvae at about the same time on successive nights over a period of about two weeks (Ennis 1973, 1975; Branford 1978; Moller and Branford 1979). Only one report documents the timing of larval release among palinurid lobsters. In contrast to the homarid lobsters, larval release occurs at sunrise for the Australian rock lobster *Jasus edwardsii* (MacDiarmid 1985). These results suggest that lobsters may possess a circadian rhythm in larval release.

In Chapter 2 of this dissertation, I investigate the hatching rhythms for both *P. argus* and *P. guttatus* to determine the phase relationship between larval release and the

LD and tidal cycles. This chapter is the preceding work for the next three chapters, in which I develop a conceptual model for egg hatching in the spiny lobster, *Panulirus argus*.

1.4 Control of Egg Hatching

Studies describing larval release behaviors have led to the development of a general model for larval release in decapod crustaceans (Forward and Lohmann 1983; Rittschof et al. 1985, 1989). The actual timing of hatching may be controlled by either the female or the embryos themselves, depending on the species and habitat (Forward 1987). To date, control of hatching time in decapods has been well-studied only in the subtidal xanthid crab *Rhithropanopeus harrisi*. Forward and Lohmann (1983) demonstrated that in *R. harrisi*, embryos control the time of hatching and suggested that the role of the female is to synchronize embryonic development. Embryos removed from the egg mass hatched at the same time as those present in the intact sponge. Yet, hatching synchrony deteriorated as the interval between removal and hatching increased. Similarly, in the semi-terrestrial crab *Sesarma haematocheir*, the success of hatching of detached eggs depends upon the time that embryos are removed from the female (Saigusa 1992). Embryos removed from the females for periods > 48 h do not hatch at all, suggesting that the female initiates the hatching process, as well as enhances the synchrony of hatching between embryos (Saigusa 1992). Saigusa (1992, 1993)

concluded that hatching and embryonic development are separate processes that are controlled independently.

In some crab species, substances associated with the embryos are released near the time of hatching and induce ovigerous females to perform stereotyped larval release behaviors, which ensure that all larvae in the egg mass are released near the same time (Rittschof et al. 1985, 1989; De Vries and Forward 1991a; Saigusa 1992, 1993; Tankersley et al. 2002). At the exact time of hatching, pheromones are either secreted by the embryos or generated by proteolytic digestion of egg membranes by enzymes released from the embryos. The female responds to the pheromones by actively pumping her abdomen. Pumping mechanically disrupts the eggs, helps to synchronize hatching, and propels larvae into the water column (Rittschof et al. 1990).

De Vries et al. (1991) suggested that embryos of subtidal crabs release enzymes that degrade the inner membrane of the egg case to produce a heterogeneous group of small peptides (< 500 Da) that are used for communication between the female crab and the larvae at the time of hatching (Forward et al. 1987). Once a few embryos hatch, these peptides are released from the egg, which cause the female to undergo her larval release behavior involving vigorous pumping of the abdomen. This action helps to break the outer membranes of the other eggs releasing more of the cue, and results in the synchronized release of larvae (Forward and Lohmann 1983). Thus, the embryos initiate hatching and females help to synchronize it.

Peptides are ideal signal molecules having many advantages as specific behavioral cues in the marine environment (Rittschof 1980a, b; Rittschof et al. 1984; Rittschof 1993; Decho et al. 1998; Rittschof and Cohen 2004). The charged nature of the amino terminus and carboxylic acid groups at neutral pH make these substances water soluble. Marine organisms possess the amino acid structural units, enzymatic 'machinery', and DNA templates to create peptides. Also, exoproteases located either intra- or extracellularly are capable of rapidly degrading peptides into amino acids to terminate a signal (Decho et al. 1998).

In Chapters 3, 4, and 5, I examine the mechanisms of egg hatching by investigating the methods of chemical communication between the eggs and the female. From this work, a conceptual model for egg hatching and larval release was developed for *Panulirus argus*.

1.5 Phyllosoma Swimming Rhythms

In the marine environment, water currents move in different directions at different depths. Thus, the vertical position of phyllosoma larvae can be of particular significance to their dispersal. To maintain their vertical position in the water column, larvae may possess behavioral responses to exogenous factors as well as ontogenetic changes in behavior, anatomy, and physiology (Forward 1974, 1976a, b, 1988; Sulkin 1984; Forward and Buswell 1989). Many larvae migrate vertically in the water column on a diel schedule due to an endogenous rhythm and/or specific behavioral responses to

environmental cues (Forward and Tankersley 2001). For example, the first larval stages of many brachyuran crabs share common behavioral traits that promote movement to the surface and maintenance of a position high in the water column (Sulkin 1973, 1975; Forward and Costlow 1974; Latz and Forward 1977; Sulkin et al. 1980) enhancing their initial dispersal from a hatching site. Although hydrodynamic conditions have some influence on larval vertical distribution, the primary depth regulatory mechanisms are probably under behavioral control, involving alternate periods of swimming and sinking in response to various environmental stimuli (*e.g.*, light, pressure, and gravity; Sulkin 1984; Forward 1988).

Models of larval dispersal rarely incorporate the behavior of larvae. Accordingly, previous models of phyllosoma larvae dispersal are based on the assumption that these larvae are simply passive floating objects transported by currents (*e.g.*, Austin 1972; Richards and Potthoff 1980; Jackson and Strathmann 1981; Kingsford et al. 2002). Most studies ignore the effect of diel vertical migration, navigation, or orientation on the transport of larvae (Wolanski et al. 1997).

The initial behaviors of the phyllosoma larvae will determine the direction and the rate of their dispersal in the coastal and oceanic environment. Phyllosoma larvae have a body shape compatible with drifting, and while they are generally regarded as having little or no directed horizontal swimming ability, they are capable of vertical movements. Previous studies suggest they undergo nocturnal vertical migration in the

upper 50 m of the water column (Yeung and McGowan 1991; Booth and Phillips 1994). Field studies suggest both phyllosoma and pueruli respond behaviorally to environmental cues. The vertical distribution of early-stage phyllosoma larvae of the western rock (spiny) lobster *Panulirus cygnus* places larvae at the surface at night and at depth during the day (Rimmer and Phillips 1979). Pueruli settle on collectors at night during the new moon and first quarter phases (Lyons 1980; Marx 1986). There are no studies that document the behavior of phyllosoma larvae underlying vertical migration.

While developing through a series of larval stages, the pelagic phyllosoma larvae has the potential to affect future recruitment and gene flow between distant lobster populations. Yet, the survival of the pelagic larval stages in oceanic environments is extraordinarily unpredictable. It is difficult to study the behavioral processes affecting planktonic survival and transport in the field, since larvae are in low abundance, widely dispersed, and highly cryptic (Phillips 1981). Thus, Chapter 6 of this dissertation includes a study of the vertical swimming behaviors of phyllosoma larvae under laboratory conditions.

1.6 Organization and Content of the Dissertation

The dissertation chapters are written as independent manuscripts, with some introductory material repeated in each. Chapter topics are as follows:

Chapter 2. Larval release rhythms of *Panulirus argus* and *Panulirus guttatus*

Chapter 3. Control of larval release in *Panulirus argus*

Chapter 4. Role of peptides in larval release in *Panulirus argus*

Chapter 5. Role of trypsin in larval release in *Panulirus argus*

Chapter 6. Endogenous swimming rhythms of phyllosoma larvae of

Panulirus argus and *Panulirus guttatus*

A Summary and Conclusions section appears at the end to integrate the information and ideas presented in the preceding chapters.

1.7 Implications of this Work

This information will aid in the evaluation of the importance of selective pressures involved in the evolution of larval release rhythms. Understanding the factors that control the timing of larval release can also help us understand how larvae survive their critical early life stages. Rhythmicity may have evolved to favor larval transport as well as to avoid predation.

Information on the control of larval release and early larval behavior is useful for fisheries management purposes. Davis and Dodrill (1980, 1989) studied the *P. argus* fishery in the Florida Keys, and came to the conclusion that “fishing apparently removes nearly every available adult lobster from Florida reefs every year.” Managers believe that the population inside the small-sized Dry Tortugas marine protected area (MPA) has a higher reproductive output than the larger population outside of the MPA within the Florida Keys. The Dry Tortugas area has a high proportion of large, reproductive females compared with only a few small ones in the lower Florida Keys.

Understanding the processes involved in larval release can help fisheries managers by identifying the environmental conditions that are required for successful egg hatching. The results of this dissertation are most likely generalized, and will apply to other decapods (other species of spiny lobsters, clawed lobsters, shrimp, etc.) of economic importance throughout the world. This information is crucial at a time in which most decapod species are in decline (Botsford et al. 1997).

2. Larval Release Rhythms in Palinurid Lobsters

2.1 Introduction

Decapod crustaceans display highly rhythmic patterns in the timing of larval release that are synchronized to natural periodic cycles including moon phase, tide, and time of day (see DeCoursey 1983; Forward 1987; Morgan 1995 for review), and can be triggered by physical and biological cues, including tidal fluctuations, LD regimes, temperature increases (Shirley and Shirley 1989), phytoplankton blooms (Starr et al. 1990) or by a combination of any of the above. Most hatching rhythms appear to be under endogenous control, since crustaceans continue to release their larvae near a specific time in an environmental cycle when placed under constant conditions (*e.g.*, Bergin 1981; Forward et al. 1982; Saigusa 1982, 1986; De Vries and Forward 1989; Ziegler and Forward 2005, 2006).

Rhythmicity of larval release may be a phenomenon that strongly affects the transport and dispersal of larvae for coastal and estuarine decapods (Dittel and Epifanio 1990; Pereira et al. 2000; Paula et al. 2004). Many crustaceans time their spawning to coincide with tidal cycles allowing them to take advantage of water currents to increase the chances of larvae being advected seaward (Forward 1987; Morgan 1995). The physical conditions at the time of larval release establish the initial conditions for

dispersal. Thus, when offspring enter the water column at appropriate times, dispersal may be greatly enhanced.

Synchronous release of larvae may also be a mechanism that promotes patch formation and larval aggregation in decapod crustaceans (Ritz 1972a, b; Rimmer 1980; Phillips 1981; Cobb et al. 1983; MacDiarmid 1985; Kerr and Duffus 2006). These aggregations would increase the survival of larvae due to the saturation of predators, and concentration of larval release in specific periods would assist in the separation of larval patches of different ages in order to avoid cannibalistic behavior that is common in decapod larvae (Paula 1989; Paula et al. 2004). Rhythmicity in larval release not only influences larval mortality, but may ultimately affect the recruitment of larvae to parental populations (Queiroga et al. 1994).

Patterns of larval release have been studied extensively in temperate intertidal and estuarine crustaceans (*e.g.*, DeCoursey 1979; Bergin 1981; Forward et al. 1982; Saigusa 1982, 1986, 1992; Morgan 1987a, b; Forward 1987; De Vries and Forward 1989; Morgan 1995; Yamaguchi 2001; Ziegler and Forward 2005, 2006), but are not as well known for tropical subtidal crustaceans (Paula et al. 2004), including spiny lobsters. The spatial distribution of larval stages of spiny lobster species indicates that first stage phyllosoma larvae are exported away from adult habitats (Yeung and McGowan 1991; Yeung and Lee 2002). The selective advantages for exporting larvae to offshore areas include stability of salinity and temperature, increased species dispersal and genetic

exchange between isolated habitats (Strathmann 1982; Bilton et al. 2002; Paula et al. 2004). In addition, females of the rock lobster *Jasus edwardsii* with late-stage eggs tend to aggregate in areas with strong currents (McKoy and Leachman 1982), suggesting that ovigerous spiny lobsters migrate to areas that will maximize the transport of larvae.

The Caribbean spiny lobster *Panulirus argus* is distributed along the east coast of the United States from North Carolina, to the Gulf of Mexico, throughout the Caribbean to Brazil (Williams 1984) and is a highly nomadic species that exhibits ontogenetic shifts in habitat over their lifetime (Herrnkind and Butler 1986; Jernakoff et al. 1994). In south Florida, adult lobsters migrate from Florida Bay to the outer reefs for mating, spawning, and larval release (Davis 1977; Davis and Dodrill 1980, 1989; Kanciruk 1980). The spawning population in Florida is concentrated in coastal reefs close to the dispersive influence of the Florida current, which dominates flow in the Straits of Florida offshore of the Florida Keys and connects the Gulf of Mexico Loop Current to the Gulf Stream (Yeung and Lee 2002). Phyllosoma larvae are oceanic plankton for 5-12 months before metamorphosing into post-larval pueruli, which undertake an onshore migration to settle in nearshore macroalgal habitats within Florida Bay (Herrnkind and Butler 1986, 1994). Pueruli metamorphose into benthic juveniles that live in vegetated habitats where they are solitary and cryptic, whereas older juveniles ("post-algal" stage) dwell gregariously under sponge and coral crevice shelters associated with hard-bottom

communities (Marx and Herrnkind 1985; Herrnkind and Butler 1986; Childress and Herrnkind 1996).

In contrast, the spotted spiny lobster *Panulirus guttatus* is a relatively small-sized, sedentary species found throughout south Florida, the Caribbean, Bermuda and Brazil (Robertson and Butler 2003). Although co-occurring with *P. argus*, *P. guttatus* is a habitat specialist (Sharp et al. 1997). All benthic stages of *P. guttatus*, from postlarvae to adult, are restricted to shallow coral reef habitats or rocky rubble in depths of 3 m or less (Sharp et al. 1997; Acosta and Robertson 2003).

This study was undertaken to compare the time of egg hatching and pattern of larval release by two sympatric species of spiny lobsters that live in tropical coral reef habitats. I tested the hypothesis that the timing of larval release is triggered by physical and biological cues that will ensure larvae will be placed in a suitable environment at a time when they can best survive. The larval release rhythms indicate that *P. argus* releases larvae near the time of nocturnal high tide whereas *P. guttatus* releases larvae near the time of sunrise independent of the time of the tides.

2.2 Materials and Methods

2.2.1 Collection and Maintenance of Animals

During the summers of 2005 and 2006, ovigerous *Panulirus argus* and *P. guttatus* were collected from coral reefs (depth 3-10 m) located near Long Key, Florida (24° 49.5

N, 80° 48.8 W) using SCUBA. Animals were immediately transported to the Keys Marine Laboratory (KML), where all laboratory experiments were conducted.

Spiny lobsters with early-stage embryos (> 10 d before hatching) were placed into individual caged enclosures in the water near KML. Individual enclosures (120 cm x 100 cm) were at least 2 m apart, and were made from PVC and plastic mesh (1 cm) which allowed the free flow of water. Placement of lobsters in enclosures allowed easy access to them while continuing to expose them to natural tidal and LD cues. The enclosures were checked daily, and egg masses were visually inspected to determine the stage of embryonic development. While in the enclosures, lobsters were fed frozen shrimp and squid daily at random times. Preliminary experiments found that the time of larval release by spiny lobsters collected directly from the field and those from these enclosures had the same relationship to environmental cycles (data not shown).

2.2.2 Embryo Development

For the purposes of this dissertation, I will define the following terms, used throughout. A fertilized egg is defined here as an 'embryo'. Embryos are attached to the maternal ovigerous hairs and are encapsulated in protective membranes which are referred to as the 'embryo case'. 'Egg mass' is defined as the entire brood of embryos attached to the ovigerous hairs on the pleopods on the abdomen of the female. 'Egg case' refers to the embryo envelope that is remaining after a larva has hatched.

A scheme for staging embryo development was constructed by observing the complete developmental sequence of the embryos from oviposition to hatching. Ovigerous females with early-stage embryos were held in the laboratory at constant temperature (27 °C) and photoperiod (14 h light: 10 h dark cycle). The developmental stages of the embryos for *P. argus* were determined by microscopic analysis to catalog the ontogeny of the different stages (Helluy and Beltz 1991). A small set of embryos (≈ 20) was removed from the surface of the egg mass of females ($n = 24$) at daily intervals. Cleavage, yolk content, eyespot development, and chromatophore formation were recorded. The number of days from the appearance of each developmental stage to hatching was determined (Table 1). Since environmental factors can control the speed of embryo development, this provided only a general estimate of the time to hatching for each developmental stage.

2.2.3 Determination of the Time of Larval Release

The developmental stages of the embryos for *Panulirus argus* were determined by microscopic analysis using a staging index described in the previous section (Table 1). When the egg mass appeared dark in color, a small set of embryos (≈ 20 eggs) was removed from the surface of the egg mass of females within the enclosures to estimate the time until hatching. Ovigerous lobsters with late-stage embryos (~ 3 days from

Table 1: Embryonic development of *Panulirus argus* in the laboratory at 27 °C

Stage	Egg Mass Color	Description	Duration of Stage (days)
I	Bright Orange	Cell division not evident; yolk granules throughout.	≈ 2-3
II	Orange	Cleavage visible; blastomeres present.	2
III	Orange	Yolk-free, transparent streak at animal pole, near funiculus; clustering of cells into segments; yolk central.	2
IV	Orange	Eyespots crescent-shaped slivers; structure of appendages visible; yolk occupies 66% embryo area.	2
V	Dark Orange	Eyespots triangular; segmentation visible around eyespots; yolk at vegetal pole occupies > 33% embryo area.	1
VI	Dark Orange	Eyespots oval shaped; appendages appearing; two clusters of yellow yolk located near center.	2
VII	Orange-Brown	Eyespots round; appendages well-formed and twitching, with red chromatophores at tips; heartbeat apparent.	2
VIII	Dark Brown	Eyespots “lima bean” shaped; fully formed larva; no yolk globules; heartbeat arrhythmic; hatching imminent.	1

hatching) were brought into the laboratory and maintained in individual glass aquaria (100.5 cm L x 25.4 cm W) containing 75 l of 0.5 μm filtered seawater (salinity of 35 - 36). For all experiments, the water was continuously aerated and changed at random times each day to remove metabolites. All experimental animals were maintained in an environmentally controlled room at 27° C and were not fed.

Lobsters were then placed under either of two different lighting conditions. To determine the solar day rhythm (see next section), one group of lobsters were exposed to the ambient LD cycle while the room lights (cool white florescent lamps; intensity = 0.76×10^{15} photons $\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$) were on during the day. These lights were extinguished at night in which lobsters were exposed to continuous low-level red light ($\approx 2 \times 10^{14}$ photons $\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$). Since crustaceans are generally insensitive to red light (*e.g.*, Forward and Cronin 1979; Cronin and Forward 1988), the lobsters were considered to be in darkness. A second group was placed under constant conditions (to determine the free running rhythm), in which the lobsters remained under continuous low level red light.

Lobsters were observed with a time lapse video system (Panasonic model 13050 VHS time lapse video recorder, and RCA model TC 1005 camera). The time of larval release was determined by reviewing the video tape and observing the larval release behaviors of the female, which consisted of rapid flexing and extension of the abdomen while actively beating the pleopods. The mean time of larval release was determined by observing the time that the female first began releasing larvae and the time that she

stopped her larval release behaviors, then determining the average between the two times.

2.2.4 Solar Day and Free Running Rhythm in Larval Release

To observe the pattern of larval release relative to the ambient LD cycle (solar day rhythm), ovigerous lobsters with late-stage embryos were placed into the environmentally controlled room in the presence of an ambient LD cycle. Preliminary results indicated that egg hatching occurred on two or three consecutive evenings for *P. argus*, whereas hatching for *P. guttatus* was limited to one night. To determine if individual *P. argus* retained unhatched embryos, the pleopods were visually inspected the morning after the first hatching event occurred. If embryos were still present, then the lobster was placed in a new aquarium with filtered seawater, and larval release was monitored each successive night until all embryos hatched. The time of larval release was then recorded (as described above) and the solar day rhythm was determined. Although hatching for *P. argus* occurred over two or three nights, only the mean time of the first hatching event was used in the statistical analysis (see section 2.2.6).

To determine the free running rhythm in larval release, lobsters of both species with early-stage embryos were placed under constant conditions (constant dim red light) for more than 10 days. The time of larval release was then recorded (as described below) and the free-running rhythm was determined.

2.2.5 Circadian Rhythm in Larval Release

To test the hypothesis that the larval release rhythm is controlled by a circadian clock entrained to the LD cycle, spiny lobsters with early-stage embryos (collected directly from the field) were randomly assigned to one of two light treatments. One treatment group was subjected to a 14:10 LD cycle similar to the ambient photoperiod. The second treatment group was subjected to a 14:10 LD cycle that was advanced by 12 h relative to the ambient photoperiod. Spiny lobsters were exposed to these photoperiod treatments for at least 10-12 days before being placed in constant conditions (constant temperature, salinity, low level red light). The onset of light began at 06:00 h in the ambient photoperiod treatment, and at 18:00 h in the advanced photoperiod treatment. Light during the day phase came from overhead cool white fluorescent lamps (0.76×10^{15} photons \cdot cm $^{-2}$ \cdot sec $^{-1}$).

Embryos from each lobster were inspected daily to determine the stage of development. When the egg mass appeared dark brown in color, a small sample of eggs (≈ 20) was removed and examined under a dissecting microscope. Lobsters that were expected to release within 48-72 h (embryo stages VI-VIII) were transferred to constant conditions at the onset of the night phase of the LD cycle and monitored under low-level red light. Only lobsters that were maintained under constant conditions for > 48 h prior to larval release were included in the study.

2.2.6 Data Analysis

The synchrony between the time of larval release and the phase of the LD and ambient tidal cycles was determined using circular statistics (Batschelet 1981). Each observation (*i.e.*, the time of larval release from one spiny lobster) was converted to a corresponding angular value that indicated the time of hatching relative to the LD or the tidal cycle. For the LD cycle, each observation was standardized for a 14:10 LD cycle, with 0° corresponding to midnight (00:00 h), and 90° corresponded to the time of sunrise (06:00 h). Thus, the 24 h LD cycle was split such that each hour was 15° apart.

Individual larval release times were used to calculate a mean time of release for each treatment group. For *P. argus*, only the time of larval release on the first night of hatching was used in the analysis. The Rayleigh test was used to determine whether hatching was synchronous (*i.e.*, differed significantly from a uniform distribution) (Zar 1999). In addition, an *r* value, which is a measure of dispersion, was calculated. The *r* values can range from 0 to 1, with higher values indicating that the data are concentrated at the same direction (Zar 1999).

If light was capable of entraining a circadian rhythm, then the mean angles (mean time of larval release) for the ambient LD cycle and the shifted LD cycle were expected to differ by 180°. A *V*-test was used to test the null hypothesis that the mean angle for the ambient photoperiod cycle trial is equal to the mean angle for the trial with a phase shifted photoperiod cycle + 180° (Zar 1999). In other words, this test compared

the ambient photoperiod (observed angle) and the shifted photoperiod experiments ($180^\circ +$ observed angle) to test whether they were the same or statistically different.

To determine if a tidal rhythm was present, the time of larval release was compared to the time of nocturnal high slack water (HSW) at the collection site on the first night that hatching occurred. One tidal cycle at the collection site (with semi-diurnal tides) has a period of about 12.4 h, which was converted into 360° . Thus, for the tidal cycle, 0° corresponded to the time of expected nocturnal HSW on the night of release and 180° the time of low slack water (LSW). Mid-ebb and flood tide were assigned values of 90° and 270° , respectively. Tidal times corresponded to the tidal cycle at Tennessee Reef near Long Key, FL (site of collection for *P. argus*) or for Coral Gardens patch reef (site of collection for *P. guttatus*). The times of the expected tidal cycles at the collection site were generated using Tides and Currents[®] (Nautical Software). A Rayleigh test was used to determine whether the times of egg hatching differed significantly from a uniform distribution relative to the tidal cycle (Zar 1999).

Since *P. argus* releases larvae on 3 consecutive nights, the time series for the observed larval release rhythm of each *P. argus* under constant conditions were analyzed for periodicity using a combination of autocorrelation and maximum entropy spectral analysis (MESA) (Dowse and Ringo 1989). After the autocorrelation coefficients (calculated at 30 min intervals) were plotted as a function of lag, period lengths were

verified with MESA according to Levine et al. (2002). Period lengths were averaged from the MESA calculations for replicate lobsters.

2.3 Results

2.3.1 Larval Release over Consecutive Nights

Panulirus argus releases groups of larvae near the time of nocturnal high slack water (HSW) over consecutive nights (Figs. 1 and 3). This hatching pattern suggests that embryos within the same egg mass develop at different rates. To test the hypothesis that all embryos within an egg mass develop asynchronously, I examined embryos taken from three discrete areas of the egg mass relative to the female abdomen: (1) innermost embryos (closest to the carapace), (2) mid-section, and (3) the periphery (outermost area of the egg mass). Microscopic inspection of developing embryos within *P. argus* egg masses ($n = 24$) suggested that all embryos within the egg mass do not develop synchronously. Embryos located on the inside of the egg mass had eye spots that were slightly less developed than those on the periphery. Interior embryos were \approx 1-2 day behind in development compared to embryos located on the exterior of the egg mass. These differences are the reason that different cohorts of larvae hatched on consecutive nights (Fig. 1).

2.3.2 Solar Day Rhythm

Female *P. argus* subjected to a 14:10 LD cycle in the laboratory release a large burst of larvae during a 20-30 min interval, and very few larvae hatch before or after the interval. Egg hatching was repeated at a similar time each night for up to three consecutive nights (Fig. 1). The number of hatching events is related to the carapace size of the parent lobster (Fig. 2). As carapace size increases, the number of consecutive hatching events also increases. The successive release of larvae each night suggests that there is an internal timing mechanism controlling larval release. Larval release for *P.*

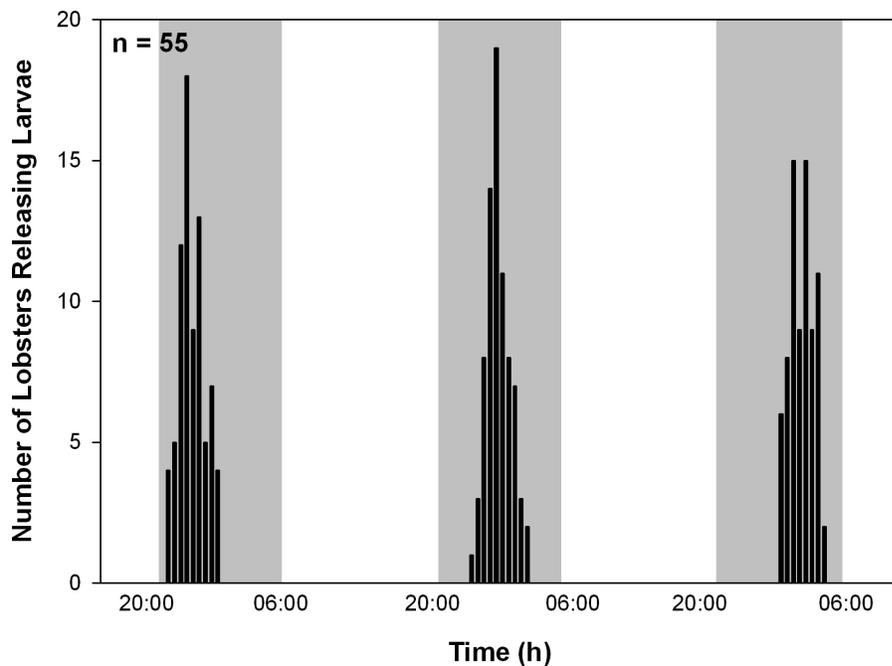


Figure 1: Mean times of larval release for individual *Panulirus argus* females on consecutive nights when exposed to a LD cycle. The shaded areas represent the night phase, while the white areas represent the day phase. Each lobster ($n = 55$) released larvae on three consecutive nights.

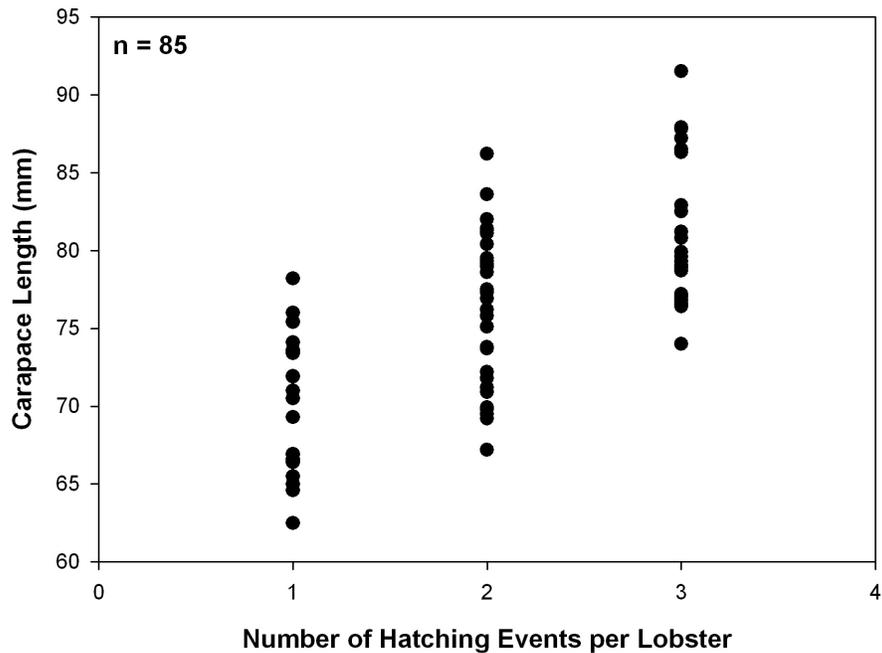


Figure 2: Carapace size of ovigerous *Panulirus argus* relative to number of hatching events on consecutive nights.

argus occurs during the dark phase of the LD cycle, and was concentrated near the time of HSW in the field (Fig. 3). A few individuals released their larvae during the light phase, in the hours immediately following sunrise.

In contrast, all hatching for *P. guttatus* occurs over a single night. Hatching in *P. guttatus* occurred between the hours of 02:00 and 10:00. *P. guttatus* displayed a solar day rhythm in which hatching was concentrated near the time of sunrise (Fig. 4). Larval release was not related to the tidal cycle.

2.3.3 Hatching under Constant Conditions Relative to the LD cycle

Hatching was observed for both *P. argus* and *P. guttatus* over a 2 - 3 week period. Any relation to tides and the LD cycle did not result simply by chance, but rather, hatching occurred at a particular time relative to the tidal and LD cycle. Ovigerous *P. argus* subjected to constant conditions for 10 - 12 days expressed a circatidal rhythm in larval release. Hatching occurred between the time of sunset and sunrise, near the time

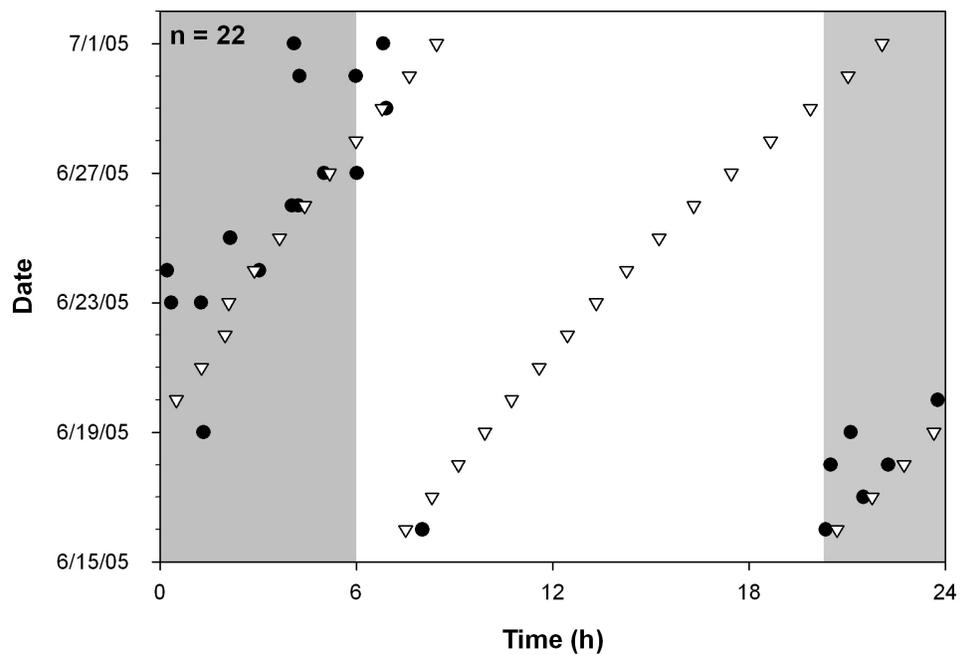


Figure 3: Mean times of larval release for ovigerous *Panulirus argus* while exposed to the ambient LD cycle. Shaded areas represent the night phase, while the white area represents the day phase. The time of larval release on the first night of release is plotted (black circle). Each point represents the time of release from one lobster ($n = 22$), while open triangles (∇) represent the time of high slack water (HSW) in the field.

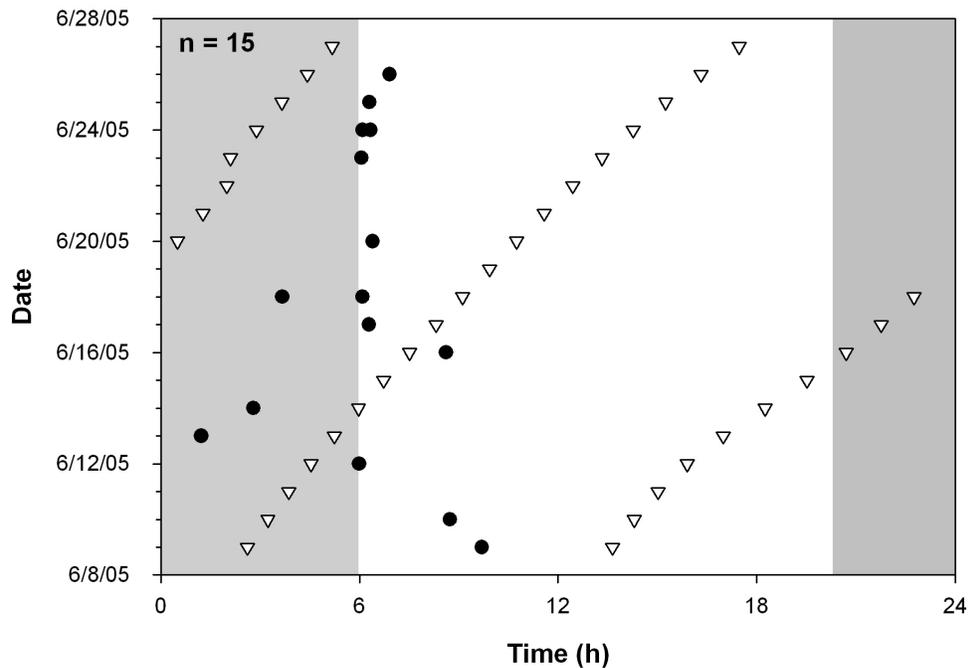


Figure 4: Mean times of larval release for ovigerous *Panulirus guttatus* while exposed to the ambient LD cycle. Shaded areas represent the night phase; white areas represent the day phase. Black circles represent the time of larval release for one spiny lobster ($n = 15$), while open triangles (∇) represent the time of high slack water (HSW).

of HSW, with a few hatching events during the light phase in the early morning hours (Fig. 5, $n = 26$). *P. argus* displayed a tidal rhythm with an average free running period of 24.6 h (S.D. = 0.93 h), which is close to the period length of a 24.8 h tidal cycle (Fig. 5). The circatidal rhythm was displayed only over the night phase of the LD cycle. Thus, lobsters release larvae at only one of the two HSW's that occur each day in this semi-diurnal tidal regime.

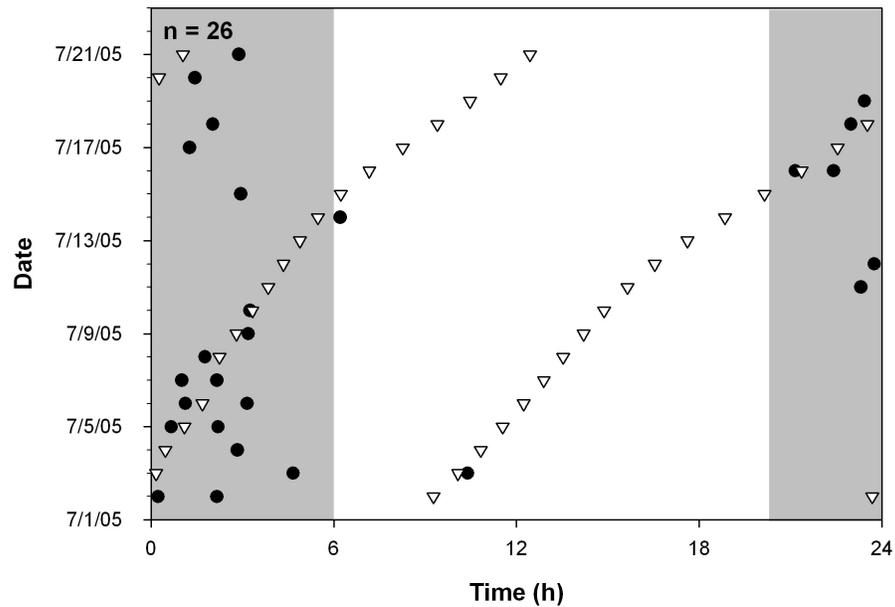


Figure 5: Mean times of larval release for ovigerous *Panulirus argus* under constant conditions for 10 – 12 days prior to larval release. Shaded areas represent the night phase of the ambient LD, with white areas representing the day phase. Each lobster ($n = 26$) contributed one data point that was the mean time of larval release on the first night of larval release. Open triangles represent the time of high slack water (HSW).

In contrast, ovigerous *P. guttatus* subjected to constant conditions expressed a free-running circadian rhythm in which hatching was concentrated near the time of sunrise (Fig. 6). The mean time of hatching was 0.80 h before the expected onset of the light phase (Fig. 7, $\bar{a} = 78.0^\circ$, $r = 0.896$, $P < 0.001$, $n = 15$).

2.3.4 Hatching under Constant Conditions Relative to the Tidal Cycle

The same data for the timing of larval release under constant conditions were replotted relative to the tidal cycle. The times of larval release for ovigerous *P. argus* placed under constant conditions were not random relative to the time of HSW (Figs. 5

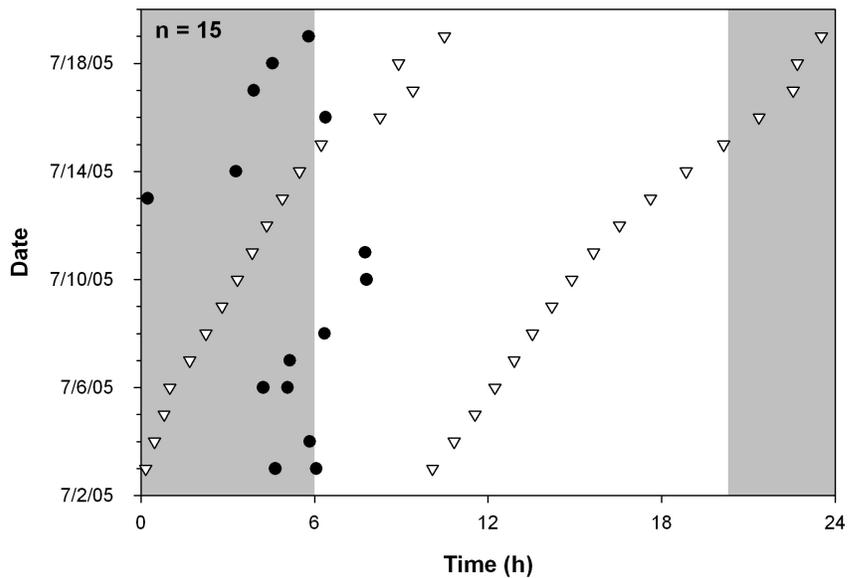


Figure 6: Time of larval release for ovigerous *Panulirus guttatus* placed under constant conditions. Shaded areas represent the night phase of the LD cycle in the field, with white areas representing the day phase. Each lobster contributed one data point. Open triangles represent the time of high slack water (HSW).

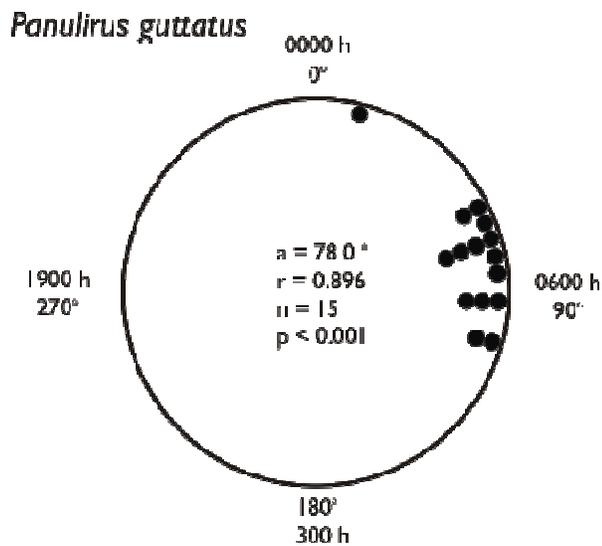


Figure 7: Mean time of larval release relative to the LD cycle for ovigerous *Panulirus guttatus* under constant conditions. Each point represents the time of release of one spiny lobster. The onset of light in the field is 06:00 h or 90°.

and 8a, $r = 0.933$, $P < 0.001$, $n = 26$). The distribution was significantly different from a uniform distribution, indicating that a circatidal rhythm in larval release was present. The mean time of larval release was 1.06 h after the time of HSW (Fig. 8a).

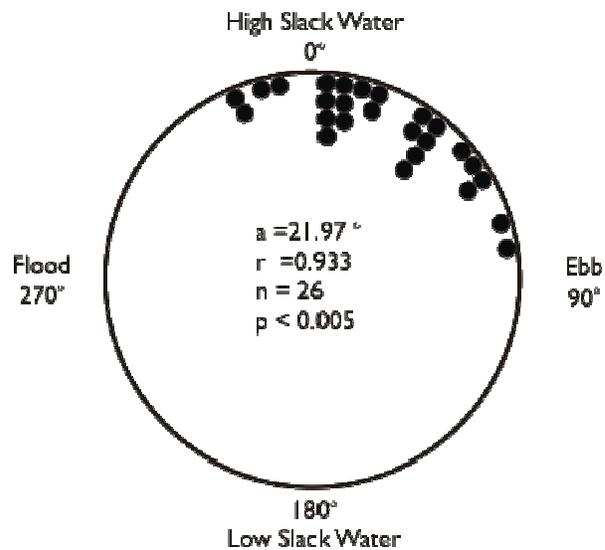
In contrast, the times of larval release for ovigerous *P. guttatus* were random relative to the tidal cycle (Figs. 6 and 8b, $r = 0.201$, $P > 0.05$, $n = 15$). The distribution was not significantly different from a uniform distribution. Thus, no circatidal rhythm in larval release was observed for *P. guttatus*.

2.3.5 Hatching under an Altered LD Cycle

P. argus subjected to a LD cycle in the laboratory for 10 - 12 d that mimicked the natural photoperiod before being transferred to constant conditions released larvae between the times of 21:00 and 09:00 (Fig. 9). 86 % of all hatching events occurred during the night phase. Hatching was also concentrated near the time of HSW in the field (Fig. 9, $\bar{a} = 33.19^\circ$, $r = 0.841$, $P < 0.005$, $n = 21$), indicating the presence of a circatidal rhythm that displays during the night phase of the LD cycle.

For comparison, a second group of *P. argus* was subjected to a 14:10 LD cycle that was advanced by 12 h (relative to the natural photoperiod treatment) for 10 - 12 days before being moved into constant conditions. This group entrained their larval release rhythm to the altered LD cycle (Fig. 10). 60 % of the hatching events occurred

A. *Panulirus argus*



b. *Panulirus guttatus*

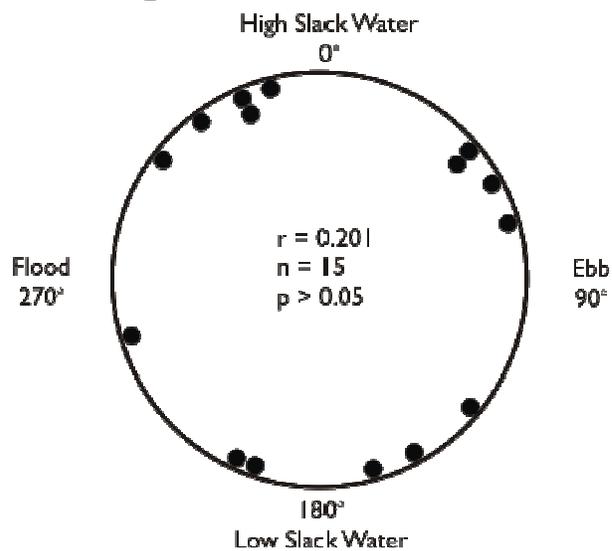


Figure 8: Mean times of larval release for *Panulirus argus* and *Panulirus guttatus* relative to the tidal cycle in the field for ovigerous females under constant conditions. Each point represents the time of release of one spiny lobster. The time of the expected phases of the 12.4 h tidal cycle are: high slack water = 0°, ebb = 90°, low slack water = 180°, and flood = 270°.

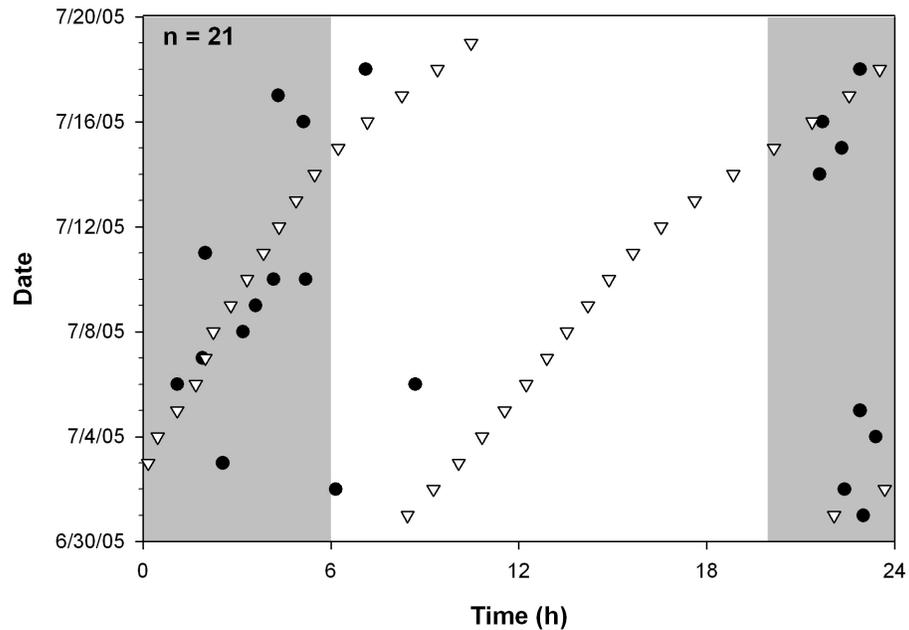


Figure 9: Mean times of larval release for ovigerous *Panulirus argus* subjected to an ambient 14:10 LD cycle and then placed under constant conditions. Shaded areas represent the night phase of the LD cycle during the entrainment period (10 - 12 days), with white areas representing the day phase. Each lobster contributed one data point that was the mean time of larval release on the first night of larval release. Open triangles show the time of high slack water in the field.

between 05:00 and 14:00 h, during the expected dark phase of the shifted LD cycle.

Larval release occurred at a mean time of 1.24 h before the time of the beginning of the shifted light phase (Fig. 10, $\bar{\alpha} = 296.4^\circ$, $r = 0.855$, $P < 0.0001$, $n = 25$). This is evidence that the LD cycle is an entrainment cue for the circadian rhythm in larval release. The tidal component was also apparent, as *P. argus* tended to release near the time of HSW during the subjective night (Fig. 10, $\bar{\alpha} = 25.78^\circ$, $r = 0.967$, $P < 0.0001$). The results of a *V*-test indicated that the mean time of release for *P. argus* in the ambient photoperiod (Fig. 9)

was not significantly different when the phase shift was taken into account (Fig. 10, $\bar{a} + 180^\circ$, $\mu = 6.16$, $P > 0.05$).

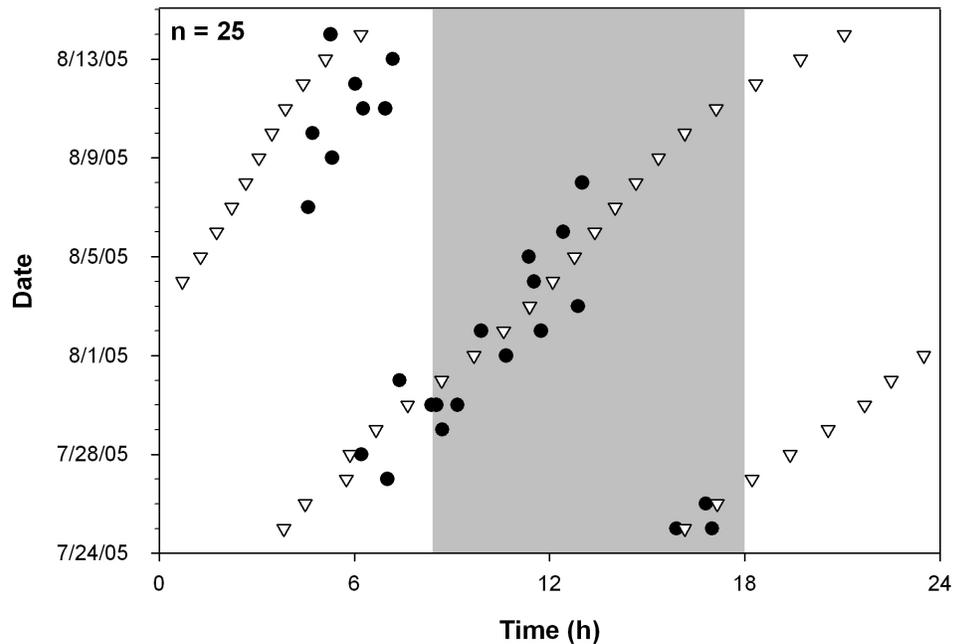


Figure 10: Mean times of larval release for ovigerous *Panulirus argus* subjected to an altered LD cycle and then placed under constant conditions. The onset of darkness (08:00 h) was advanced by 12 h relative to the ambient LD cycle treatment. Shaded areas represent the night phase of the LD cycle during the entrainment period (10 - 12 days), with white areas representing the day phase. Each lobster contributed one data point that was the mean time of larval release on the first night of larval release. Open triangles show the time of high slack water in the field.

Two groups of *P. guttatus* were also subjected to either an ambient 14:10 LD cycle or an altered cycle that was shifted by 12 h (relative to the ambient photoperiod treatment) for 10 - 12 days. Hatching for *P. guttatus* subjected to an ambient LD cycle occurred near the time of expected sunrise, with a mean time of 2.7 h after the onset of light (Fig. 11, $\bar{a} = 83.7^\circ$, $r = 0.84$, $P < 0.005$, $n = 12$). When ovigerous *P. guttatus* were subjected to an altered LD cycle, the time of larval release was also shifted (Fig. 12, $\bar{a} =$

274.2°, $r = 0.88$, $P < 0.001$, $n = 17$), indicating that the LD cycle was the synchronizing agent for the circadian rhythm in larval release for *P. guttatus*. The mean time of release for *P. guttatus* under a natural photoperiod (Fig. 11) was not significantly different from the shifted photoperiod (Fig. 12, $\bar{a} + 180^\circ$, $\mu = 0.373$, $P > 0.05$). Thus, both species of spiny lobsters were able to entrain to the LD cycle that they experienced in the laboratory.

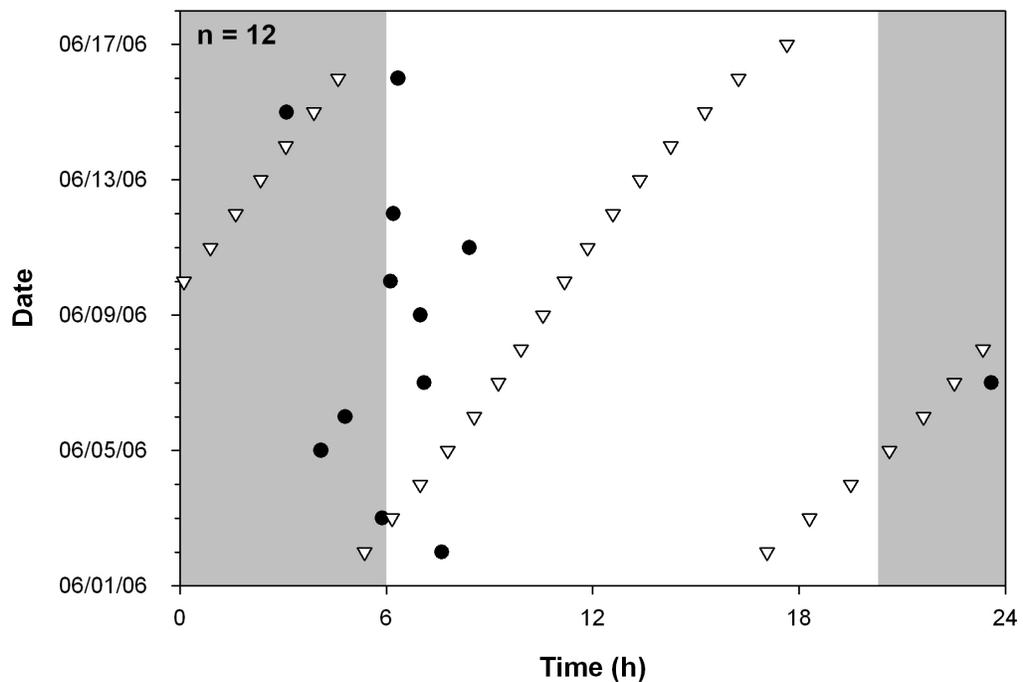


Figure 11: Mean time of larval release for ovigerous *Panulirus guttatus* subjected to an ambient 14:10 LD cycle and then placed under conditions. Shaded areas represent the night phase of the LD cycle during the entrainment period (10 - 12 days), while the white area represents the day phase. Open triangles represent the time of high slack water in the field.

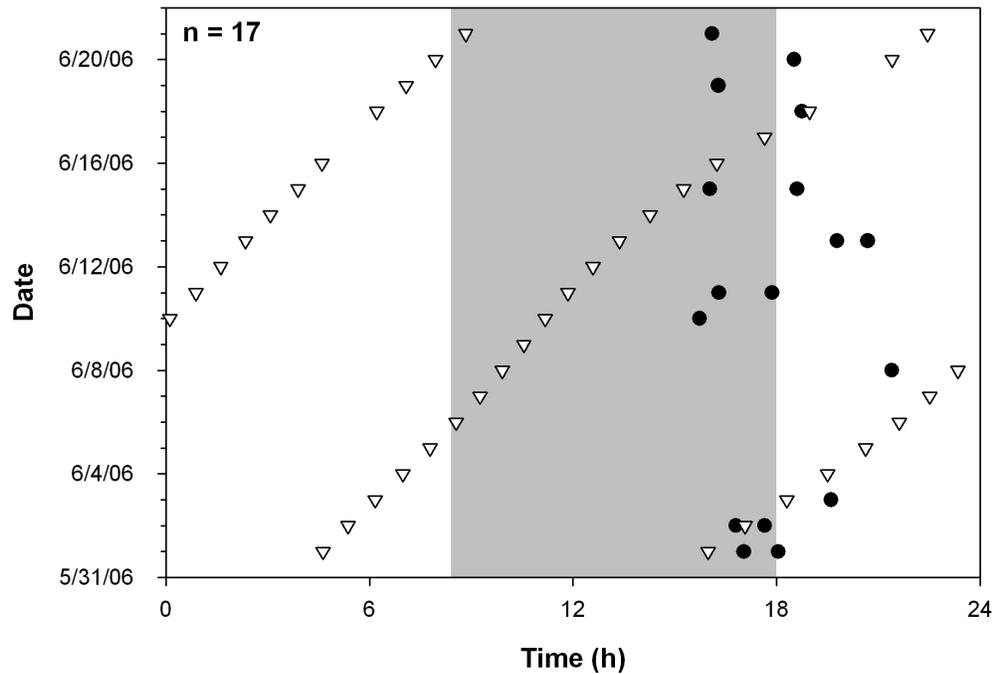


Figure 12: Mean times of larval release for ovigerous *Panulirus guttatus* subjected to an altered LD cycle and then placed under constant conditions. The onset of darkness (08:00 h) in the altered LD cycle was advanced by 12 h relative to the ambient LD cycle treatment. Shaded areas represent the night phase of the LD cycle during the entrainment period (10 - 12 days), with white areas representing the day phase. Open triangles represent the time of high slack water in the field.

2.4 Discussion

Panulirus argus and *P. guttatus* both display rhythms in larval release under constant conditions in the laboratory for 10-12 days when isolated from any LD or tidal cues. Larval release is concentrated around the times of nocturnal HSW at the collection site for *P. argus*. For *P. guttatus*, the majority of hatching events occurred near the time of expected sunrise. Larval release for both *P. argus* and *P. guttatus* is precise, as it occurs over a short, specific time and lasts several minutes. These lobsters actively take part in egg hatching, as they exhibit stereotypical larval release behaviors, including the

rapid flexing and extension of the abdomen and vigorous beating of the pleopods. The clawed lobster *Homarus gammarus* also must actively beat pleopods to help break open the egg case and free larvae (Branford 1978). This synchronous behavior may be controlled by the female (Branford 1978; DeCoursey 1979) or by the developing embryos (Pandian 1970; Ennis 1973; Forward and Lohmann 1983). The clock controlling hatching rhythmicity for *P. argus* is the developing embryos and not the female (Forward et al. 1986; Chapters 3 and 4).

P. argus displayed a nocturnal circatidal rhythm with an observed free-running period length of 24.6 h, which is in between the 24 h length of a solar day and the 24 h 50 m period of a lunar day. If this was a circadian rhythm, one would expect to see a period length closer to 24 h for lobsters under the ambient and shifted LD cycle treatments, since a Zeitgeber was present for the endogenous clock to entrain to. This was not the case, as release times progressed through the night and occurred near the time of HSW, even under a LD cycle. Under the shifted LD cycle (Fig. 10), the release of larvae by *P. argus* occurred near the time of daytime HSW in the field, indicating that a circadian rhythm is not the sole rhythm being displayed. A similar situation occurs for the subtidal crab *Neopanope sayi*. This crab releases larvae preferentially near the time of nighttime high tide, suggesting that both a circatidal and a circadian rhythm are involved (De Vries and Forward 1989). This pattern of larval release may result from an interaction between two clocks, one with a circatidal periodicity and one with a

circadian periodicity, such that the expression of the output of the tidal clock depends on the phase of the circadian clock (Palmer 1995). These results suggest that larval release for *P. argus* is being controlled by two endogenous clocks, and that each clock is not expressed independently. One circadian oscillator is entrained by the LD cycle, and a circatidal oscillator that is entrained by some cue from the tidal cycle (most likely hydrostatic pressure or current), controls the larval release rhythm for *P. argus*. The circadian clock is capable of shifting the circatidal rhythm, so that larval release will occur at the time of HSW during the dark phase, indicating that there is an interaction between the clocks.

The tidal rhythm exhibited by *P. argus* persisted for 10-12 days in the absence of tidal cues, suggesting that the tidal clock must have been set near the time of oviposition, prior to advanced embryo development (Fig. 5). Rhythmic behavior in spiny lobsters is probably based on multiple physiological clock components. These rhythms are controlled by an endogenous clock that may be related to the development of the neurosecretory systems of the larvae (Naylor 1988). The timing of molting and metamorphosis in *P. japonicus* phyllosoma larvae occurs synchronously at the time around sunrise throughout all the larval phases, while metamorphosis to the puerulus stage occurs near the time of sunset (Matsuda et al. 2003). The same clock may be used by the developing embryos to assist in synchronous egg hatching.

Both species advanced their larval release rhythm when the LD cycle was advanced, indicating that the timing of the rhythm is entrained by the LD cycle (Figs. 9 and 10). For *P. argus*, ovigerous females subjected to a LD cycle that was advanced by 12 h shifted the time of larval release to the next tidal cycle (Fig. 9). This ability to shift the larval release rhythm may have an adaptive value in that the phase of the rhythm is continuously adjustable in relation to either geographic or seasonal changes (Morgan 1995).

Hatching for *P. guttatus* occurs near the time of sunrise, indicating a circadian rhythm in larval release. This release pattern is similar to the Australian rock lobster *Jasus edwardsii* (MacDiarmid 1985), in which larval release occurs at sunrise. These results suggest that other spiny lobster species may possess a circadian rhythm in larval release. In contrast, most individual *P. argus* released larvae on nighttime tides, although some daytime releases were observed (Fig. 3). Crabs that live in mid- to low-intertidal habitats such as *Eurypanopeus planus* (Christy 1986) and subtidal crabs such as *Neopanope sayi* (De Vries and Forward 1989), also release at the time of night and daytime time high tides. Selective pressures (such as predation upon larvae and adults) for decapod crustaceans to release at night seems to be greater for animals that are terrestrial or that live in the high intertidal zone, since these species usually release larvae only at night.

Typically, larval release by decapod crustaceans is reported to be a single event with all larvae released within a few minutes, as was the case for *P. guttatus* (Fig. 3). However, many exceptions exist, including our results for *P. argus* (Fig. 1). Among other species, larval release in the laboratory occurs shortly after the onset of darkness on a series of consecutive nights for the homarid lobsters, *Homarus gammarus* (Ennis 1973; Branford 1978), *H. americanus* (Ennis 1975), and *Nephrops norvegicus* (Moller and Branford 1979). Larval release in the green crab *Carcinus maenas* is repeated over several consecutive tidal and diel cycles (Zeng and Naylor 1997). Tanner crabs, *Chionoecetes bairdi*, release larvae over many consecutive events, during the first few hours after dusk (Stevens 2003). Hatching for king crabs, including *Paralithodes platypus*, *P. camtschaticus*, *Lithodes aequispinus*, and *Lithodes santolla* occurs over a period of 29 to 41 days, depending on region or temperature (Stevens 2006; Thatje et al. 2003; Paul and Paul 2001).

Releasing larvae over consecutive nights suggests that *P. argus* engages in a “bet hedging” strategy by extending their reproductive risk over time (Slatkin 1976; Stevens 2006). Bet hedging could improve mean fitness by allowing a species to produce fewer offspring per unit time rather than maximizing the number of offspring at one time. This strategy would reduce predation as well as competition between larvae by spreading their offspring in time rather than space alone (Philippi 1993). This pattern also ensures that at least some larvae will survive in the event of episodic environmental

conditions, and could help improve the species' ability to adapt to variable future environments (Stevens 2006).

The release pattern of *P. argus* is similar to the pattern among brachyuran crabs, in which hatching occurs near the time of nocturnal high tides (see Forward 1987; Morgan 1995 for review). If high tide occurs shortly before sunset, crabs delay release until the onset of darkness. Several days later, when the time of high tide occurs shortly after sunset, hatching is more closely associated with the time of high tide (e.g., Saigusa and Hidaka 1978; Bergin 1981; Saigusa 1981, 1982; Christy 1986; Forward et al. 1986). Observations by Paula (1989) and Queiroga et al. (1994) showed a similar release pattern for *Carcinus maenas* in Portugal, with peaks of larval abundance occurring during nocturnal ebb tides. These observations support Paula's (1989) conclusion that the time of high tide during the LD cycle is important in controlling the synchrony of hatching by *C. maenas* (Queiroga et al. 1994).

Several hypotheses have been proposed for the ultimate functional advantages of the observed patterns of larval release. First, larval release at the time of high tide may be an adaptation to avoid stressful or lethal salinities, since most decapod larvae are generally intolerant of low salinity conditions that occur during low tide (Costlow and Bookhout 1959; Forward et al. 1982). This is unlikely the cases for either *P. argus* or *P. guttatus*, as these lobsters are located in coastal waters in which the salinity and temperatures are relatively stable. The second hypothesis predicts that the synchronous

release of larvae lowers the risk of predation by “swamping” potential predators (DeCoursey 1983), thereby minimizing the likelihood that the progeny of one female will suffer disproportionately from predation (Paula 1989). Third, the release of larvae with the outgoing current at night enhances the chances that larvae are rapidly removed from the area, minimizing the exposure time to visual predators (abundant planktivores), thereby enhancing larval survival (Salmon et al. 1986; Morgan 1995; Morgan and Christy 1995). Selective pressures associated with predation may be the ultimate cause for the nocturnal tidal rhythm exhibited by *P. argus*. These pressures would include predation on ovigerous females by visual predators as well as predation upon larvae by planktivores. For *P. argus*, hatching at the time of HSW prior to the ebbing tide suggests that larval transport toward offshore areas for larval development is of utmost importance.

Release during the day implies that predation is of secondary importance in the evolution of this timing pattern in *P. guttatus*. Phyllosoma larvae of *P. guttatus* are positively phototactic and they possess an endogenous swimming rhythm that would facilitate their transport in the ebbing tidal currents (Chapter 6). Thus daytime release is appropriate, according to their larval behavior. Larvae move towards light, and therefore would be present in surface waters where the net flow of water is away from the female. Daytime release is not typical for crustaceans, and usually occurs in species whose larvae have evolved morphological adaptations, such as spination, crypticity, or

transparency to protect themselves from planktivorous visual predators (Morgan 1989). *P. guttatus* larvae are transparent, which may be an adequate defense against fish predation (Morgan 1987a, b). *Panulirus* spp. phyllosoma larvae are found in surface waters of the ocean in the Gulf of Mexico and the Straits of Florida (Yeung and McGowan 1991), so advection of these larvae is presumably determined by near-surface currents. Thus, rapid transport to nursery areas may also be particularly crucial for larval survival.

Nocturnal larval release near the time of HSW for *P. argus* may be an adaptation to reduce predation pressure, while also assisting the larvae with transport to appropriate nursery areas in offshore waters. This argument is supported by Morgan (1987a, b) who demonstrated that crab larvae of transported species are preferred and easily preyed upon by larval fish than species which as larvae are retained near adult habitats. As another example, hatching for brachyuran crabs in Mar Chiquita Lagoon, Argentina is rhythmic, with a sharp increase in larval abundance during nocturnal ebb tides (Anger et al. 1994), exporting crab zoea outside of the lagoon since development must take place in coastal marine waters (Anger et al. 1994). This situation is similar for *P. argus* larvae that are exported to offshore areas for development.

There may be multiple ultimate and proximate factors regulating the hatching synchrony of crustaceans, and this may be responsible for interspecific diversity of hatching patterns. The proximate causes for hatching synchrony are changes in

behavior related to the LD, tidal, semi-lunar, and lunar environmental cycles. These may have an independent, specific effect on hatch timings on lobsters and can either alone or in combination produce a variety of temporal reproductive patterns. The ultimate factors involved in the regulation of hatching synchrony include the avoidance of predators, avoiding adverse physiological conditions, and transport to nursery habitats for development (Forward 1987; Morgan 1995). Synchronous hatching likely enhances the survival of adults, embryos, or larvae during and after the time of larval release. A thorough understanding of the effect of various selection pressures on the reproductive timing of related animals is required to determine the adaptive significance of reproductive synchrony.

3. Control of Larval Release in the Caribbean Spiny Lobster, *Panulirus argus*

3.1 Introduction

Larval release in most decapod crustaceans is under endogenous control and is synchronized with environmental cycles, such as light:dark (LD), tidal, or lunar cycles (see Forward 1987; Morgan 1995 for reviews). Larval release is a brief event that is associated with specific behaviors of the female (DeCoursey 1979; Forward et al. 1982; De Vries 1990; Tankersley et al. 2002). In brachyuran crabs, embryos typically hatch all at once over a short time interval as the female prods the egg mass with her walking legs and vigorously pumps her abdomen to help break open the cases containing the embryo within (Forward et al. 1982; Christy 1986; De Vries and Forward 1989). In contrast, in anomuran crabs and lobsters, larvae are released in more than one short burst at the time of consecutive tidal phases or nights (Ennis 1973; Branford 1978; Ziegler and Forward 2005, 2006; Chapter 2). A female lobster will flex and extend her abdomen and strongly flutter her pleopods back and forth during the hatching event. Thus, the temporal control of larval release in decapods appears to vary with species.

The time of egg hatching may be controlled by the behavior of the female, by the developing embryos releasing a chemical that breaks open egg cases (membranes that hold and protect the embryo during development), or by some combination of the two. Control of larval release has been examined in the subtidal crabs *Rhithropanopeus harrisi*

(Forward et al. 1982, 1987; Forward and Lohmann 1983; Rittschof et al. 1985, 1989), *Neopanopeus sayi* (De Vries et al. 1991), and *Callinectes sapidus* (Tankersley et al. 2002). These studies have led to the development of a model for larval release for brachyuran crabs (Forward et al. 1987; De Vries et al. 1991; Tankersley et al. 2002), in which the developing embryos control the exact time of hatching, whereas the female controls the synchrony of larval release. Near the time of hatching, the female begins to exhibit larval release behaviors because she is responding to substances released from hatching embryos, termed 'pumping pheromones'. As more embryos hatch, the concentration of the pheromone increases, thereby stimulating the female to initiate more and more vigorous abdomen pumping. This pumping activity helps to physically disrupt the embryo case. More pheromone leads to more pumping, which leads to more breaking of embryonic cases, and this positive feedback system results in the synchronous release of larvae.

The timing of larval release for the Caribbean spiny lobster, *Panulirus argus*, is also precisely timed. Larval release occurs at the time of slack water after nocturnal flood tides (Chapter 2). Ovigerous spiny lobsters display stereotypical larval release behaviors similar to brachyuran crabs. The female lobster rises onto her walking legs, probes her egg mass with the tips of her walking legs and rapidly flexes and extends her abdomen while actively beating the pleopods. During larval release, this vigorous 'pleopod pumping' behavior acts to synchronize embryo hatching. Homarid lobsters

are also known to pump their pleopods during the hatching process as a physical mechanism to release larvae (Ennis 1973, 1975; Branford 1978).

The objective of this study was to investigate whether the model for larval release of subtidal brachyuran crabs can be applied to *P. argus*. Although homarid lobsters and brachyuran crabs have been studied, palinurid lobsters have not been investigated. I designed the following experiments to test the hypothesis that spiny lobster embryos contain substances (pheromones) that prompt the ovigerous females to initiate the movement of the pleopods that occurs during larval release.

The stereotypical larval release behavior (pleopod pumping) was used as a biological assay to determine if ovigerous spiny lobsters respond to (1) pheromone substances released by hatching embryos, (2) substances present in homogenates of early- and late-stage embryos, (3) pheromones released from embryos prior to hatching, and (4) substances present in homogenized membranes from post-hatch egg cases.

3.2 Materials and Methods

3.2.1 Collection and Maintenance of Spiny Lobsters

Ovigerous spiny lobsters *Panulirus argus* (Latreille, 1804) were collected by hand using SCUBA from June to August 2005 at coral reefs near the Keys Marine Laboratory in the Florida Keys, USA (24° 49.567 N, 80° 48.884 W). Lobsters were maintained in individual glass aquaria containing \approx 75 l of continuously-aerated seawater (salinity of

35 – 36; 27°C) filtered to remove particles > 5 µm. The size range of ovigerous females used in this study was from 64.3 mm to 92.1 mm carapace length. The water in the aquaria was changed daily at random times. A 14 h light: 10 h dark cycle simulated ambient conditions at the time of collection.

3.2.2 Effect of Egg Development Stage on Pleopod Pumping Activity

To determine if pleopod pumping activity of female lobsters changes with the embryonic development stage, the spontaneous pumping rate of ovigerous lobsters carrying embryos of different developmental stages was determined ($n = 119$, see Fig. 1 for replicate distribution). I classified embryos into four different groups according to characteristics based upon embryo stage (stage I – II = early, III – IV = early-mid, V – VI = mid, and VII – VIII = late; see Table 1, Chapter 2 for descriptions).

Spontaneous pumping activity was quantified because it is the behavior involved in larval release, and in other species, this pumping rate can change with the developmental state of the embryos (De Vries et al. 1991; De Vries and Forward 1991). A spiny lobster was placed in a glass aquarium (50.8 cm L x 25.4 cm W) containing 3 l of filtered seawater. After a 5-min acclimation period, I recorded the number of pleopod movements during a 1-min period. These observations were made under a dim-red light (≈ 650 nm) to prevent disturbance of lobsters by the presence of an observer. Crustaceans are, in general, insensitive to red wavelengths (Forward and Cronin 1979). All lobsters were tested within 24 h of collection and each lobster was tested once,

between 10:00 and 20:00 h, to control for the variability due to a possible endogenous rhythm. Pumping frequencies were compared using a Kruskal-Wallis test and non-parametric multiple comparison (Zar 1999).

3.2.3 Frequency of Pleopod Pumping

I hypothesized that spiny lobster embryos release a chemical cue (*e.g.*, pheromone) at the time of hatching which induces the stereotypical pleopod pumping behavior of the female. To test this hypothesis, the frequency of pleopod pumping was used as a bioassay to quantify the larval release behavior of the female. The experimental procedure was to place the ovigerous female in a glass aquarium containing 5 l of filtered ($> 0.5 \mu\text{m}$) offshore seawater. After a 10-min acclimation time, the number of pleopod movements was counted during an initial 1-min interval as a measure of spontaneous pumping activity. The lobster was then immediately transferred to an identical aquarium with a test solution and the number of movements in a 1-min period was counted after 10 min of acclimation. A positive response by a lobster was tallied if the pleopod pumping rate increased upon exposure to the test solution. Lobsters were scored as unresponsive if pumping activity decreased or stayed the same. The percent of lobsters that responded positively served as a relative measure of the biological activity of the test solution.

For each test solution, a spiny lobster was tested in a graded series of nine concentrations. The first solution in the series contained filtered offshore water

(concentration = 0 ml⁻¹) and served as a control for spontaneous pumping activity. To minimize the chances of sensory adaptation, the remaining eight concentrations proceeded from the lowest to highest (10, 20, 30, 40, 50, 60, 80, and 100 ml⁻¹). Each lobster was placed in filtered seawater (control) for at least 30 min before being retested at the next higher concentration. Thus, the exposure sequence was 10 min in offshore water, 10 min in a test solution, 30 min in offshore water, 10 min in the next highest test solution, 30 min in offshore water, and so on until all concentrations were tested. I determined by preliminary experiments that 30 min was sufficient time between exposures to test solutions for the lobster to return to the responsive state (*i.e.*, the same spontaneous pumping rate in offshore water). Repeated exposure to a specific test concentration using this procedure produced the same response. Sample sizes for each test solution ranged from 12 to 20. Responses of lobsters to test solutions were compared to responses in filtered sea water using a χ^2 -test for multiple proportions and an associated multiple comparison test (Zar 1999).

3.2.4 Effect of Hatch Water Concentration on Pumping Rate

I compared the minimum concentration that can induce a significant pumping response (threshold concentration) in lobsters with early- and late-stage embryos to determine if the sensitivity of ovigerous females to chemical cues in hatch water varies with the developmental state of the female's embryos. Spiny lobsters were separated into two groups. I designated the two groups based upon embryo stage: early- and late-

stage embryos. Early-stage embryos are defined as embryos from stage I (bright orange color, uniformly distributed yolk, no presence of cleavage or appendages) to stage III (cleavage evident and > 75% of volume filled with yolk) (Table 1, Chapter 2). Late-stage embryos were defined as embryos with well-developed eye spots, a heartbeat, appendages, and were less than three days from hatching (stage VII to VIII).

Hatch water was prepared by placing individual ovigerous lobsters into a holding tank (100.5 cm x 30.6 cm) containing filtered offshore seawater (0.5 μm , salinity ≈ 35) approximately 2-3 h prior to the predicted time of larval release which occurred around the time of slack water after nocturnal flood tide. Immediately following hatching, the female was removed from the container. Hatch water was passed through a 100 μm filter to remove the larvae, embryonic membranes, and any unhatched embryos. The titer of the pheromones in the hatch water was estimated by counting the number of larvae in 10 subsamples of hatch water. The pheromone concentration of the hatch water was expressed as larvae ml^{-1} . A uniform amount of pheromone was assumed to be released upon hatching of each embryo. Hatch water was used the day after the hatching event (within 6-8 h after hatching). Hatch water was diluted with filtered offshore water to produce the test concentrations (0 larvae ml^{-1} (control), 10, 20, 30, 40, 50, 60, 80, and 100 larvae ml^{-1}).

3.2.5 Effect of Embryo Homogenates on Pumping Rate

I measured the pumping response of ovigerous spiny lobsters exposed to increasing concentrations of water containing crushed early- and late-stage embryos to determine whether pheromone levels change with embryo development. The egg mass was carefully removed from the abdomen of an ovigerous female by cutting the setose ovigerous hairs that hold embryos. The concentration of embryos in the stock solution was estimated by weighing and counting the number of embryos in subsamples removed from the egg mass prior to homogenization. Embryos were crushed using a tissue homogenizer and the solution diluted in filtered offshore water to obtain test concentrations that ranged from 10 to 100 eggs ml⁻¹. These solutions were tested within 1 h of preparation.

3.2.6 Effect of Egg-Conditioned Water on Pumping Rate

To determine if the concentration of stimulatory substances leaching from the embryos varies with the developmental stage of the embryos, I compared the pumping response of ovigerous lobsters with late-stage embryos to water conditioned with either early- or late-stage embryos. This experiment was designed to determine if the pheromones that are responsible for stimulating larval release behaviors are released from the developing embryos into the water prior to the hatching event. Embryo-conditioned water was prepared by carefully removing an egg mass from an ovigerous lobster and placing it in a flask containing 1 l of 0.5- μ m filtered seawater. The detached

egg mass was incubated under gentle aeration for 20 h before it was removed. The embryo-conditioned water was subsequently checked for the presence of broken embryos or free-swimming larvae. If present, then the water was discarded, since it was assumed to contain pheromone substances from the broken embryos or from released larvae. Subsamples of embryo counts from the egg mass were used to quantify the concentration of chemical substances present in conditioned water. Aliquots of the stock solution were diluted with 0.5- μm filtered seawater to produce the individual test concentrations (10-100 embryos ml^{-1}).

3.2.7 Effect of Post-Hatch Egg Case Homogenates on Pumping Rate

To test whether the breakdown of the post-hatch egg cases was responsible for the release of pheromones, I measured the pumping response of ovigerous females with late-stage embryos to water containing homogenized egg cases only. The empty egg cases on a female lobster will remain attached to the pleopods for up to 24 h after a hatching event (Ziegler, personal observation). Shortly after a hatching event occurred, I carefully removed the empty cases by cutting the setose ovigerous hairs on the pleopods. Using a smooth textured brush, the cases were gently removed from the ovigerous hairs. Any unhatched embryos or unfertilized eggs were discarded. The egg cases were homogenized, and the solution was diluted in filtered offshore water. The concentration of egg cases in the stock solution was estimated by weighing the excised cases and then counting the number of egg cases in 10 subsamples prior to

homogenization. Therefore, concentrations are expressed as egg cases ml⁻¹. Since the creation of this solution was labor intensive, and since I observed that only late-stage females responded significantly to other test concentrations, I chose to only examine the response of late-stage females to this treatment.

3.3 Results

3.3.1 Spontaneous Pleopod Pumping Activity

Spontaneous pleopod pumping rates increased with increasing embryo age. Pumping rates were low for females with early-stage (mean = 2.8 pumps min⁻¹), but were similar for early-mid and mid-stage embryos (mean = 9.5 - 13.5 pumps min⁻¹) (Fig. 13a). Pumping rates increased dramatically for females with late-stage embryos (mean = 38.0 pumps min⁻¹) (Kruskal-Wallis, $H = 101.59$, $df = 3$, $P < 0.001$; Fig. 13a).

The percentage of ovigerous *P. argus* displaying spontaneous pleopod pumping activity changed with the developmental stage of the embryos. No significant difference in pumping response was observed for lobsters with early- and early-mid stage embryos (stages I – III) (Fig. 13b). These percentages were relatively low compared to the percentage of lobsters with mid- and late-stage embryos that pumped (Fig. 13b). The percentage of lobsters that pumped did not differ significantly among lobsters with mid- and late-stage embryos (stages IV – VIII; Fig. 13b; non-parametric multiple comparisons).

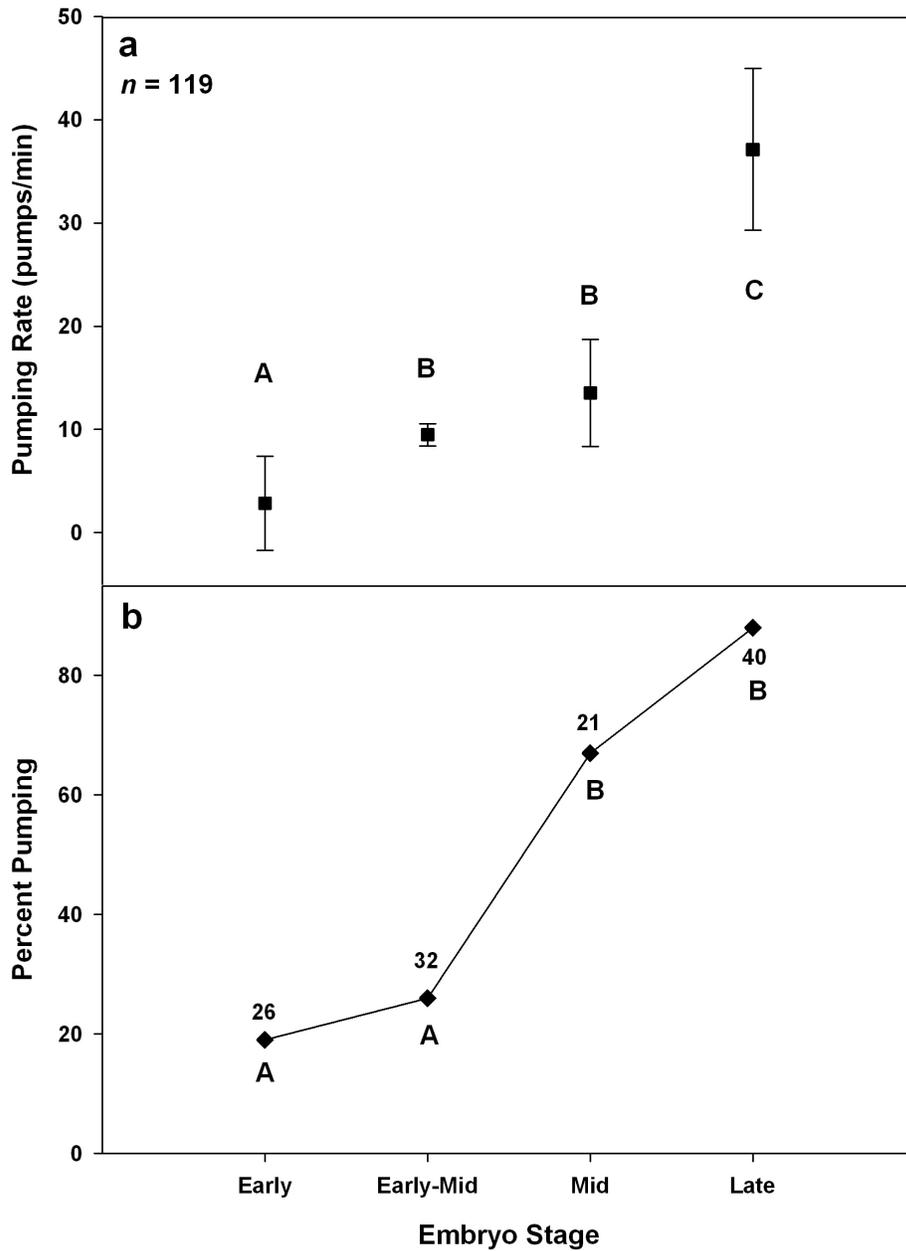


Figure 13: *Panulirus argus* spontaneous pleopod pumping rates. Frequency of pleopod pumping activity in lobsters that pumped (a) and percentage of lobsters that displayed pumping activity during a 1-min observation period as a function of developmental stage of embryos (b). Numbers beside points are sample sizes for each group. Similar letters indicate values not statistically different at $P < 0.05$.

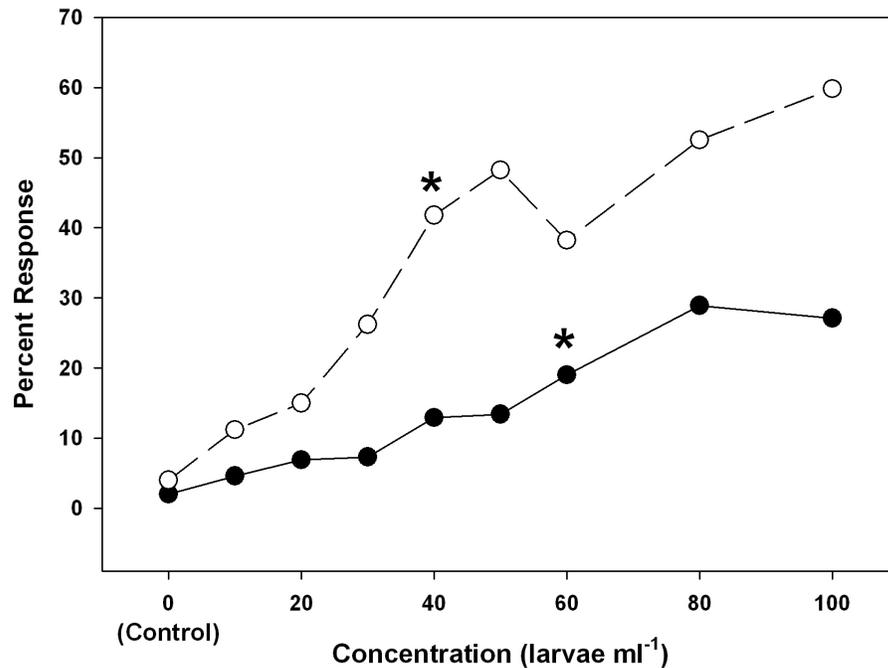


Figure 14: Effect of hatch water on pleopod pumping response. Mean percentage of ovigerous *Panulirus argus* with early- (stage I – III; solid line; $n = 20$) and late-stage (stage VII – VIII; dashed line; $n = 16$) embryos that displayed a response to different concentrations of hatch water. Lowest concentration at which pumping response was significantly different from control is indicated by an asterisk.

3.3.2 Response to Hatch Water

The pumping activity of ovigerous females with both early- and late-stage embryos increased when exposed to increasing concentrations of hatch water (Fig. 14), suggesting that pheromones stimulating abdominal pumping are released from embryos at the time of hatching. The lowest percent concentrations (threshold) that induced a response significantly different from controls were 40 larvae ml⁻¹ for lobsters with late-stage embryos ($\chi^2 = 21.97$, $df = 8$, $P < 0.005$, $n = 20$), and 60 larvae ml⁻¹ for lobsters with early-stage embryos ($\chi^2 = 15.61$, $df = 8$, $P < 0.05$, $n = 16$). As the concentration increased

above the threshold level, pumping activity increased and was significantly greater than controls (Fig. 14). Females with early-stage embryos had consistently lower pumping responses than females with late-stage embryos. This difference may be attributed to the fact that early-stage ovigerous females do not have high spontaneous pumping rates in activity (Fig. 13). Spiny lobsters without an egg mass did not show a pleopod pumping response to hatch water (Table 2), suggesting that female spiny lobsters become sensitive to the pheromone only after oviposition.

Table 2: Pumping response of non-ovigerous spiny lobsters ($n = 10$)

Species	% number of pumps/min (X)	SD
<i>Panulirus argus</i>	.04	.118

3.3.3 Response to Water Containing Homogenized Embryos

An assay was conducted with water containing homogenized early-stage embryos (stages I – III) to determine if the concentration of pheromone varies with the developmental stage of the embryos. For females with late-stage embryos (stages VII – VIII), the percent response increased steadily with increasing concentrations of early-stage embryo water (Fig. 15; $\chi^2 = 24.03$, $df = 8$, $P < 0.05$, $n = 15$) and plateaued between 20 % and 25 % concentrations at > 40 eggs ml^{-1} . There was little responsiveness by females with early-stage embryos (stages I – III) to water containing early-stage embryos (Fig. 15;

$P > 0.05$, $n = 12$), and no concentration elicited a significant response from lobsters with early-stage embryos. These results indicate that the pheromone is present in early-stage embryos but only females with late-stage embryos are sensitive to the test concentration levels.

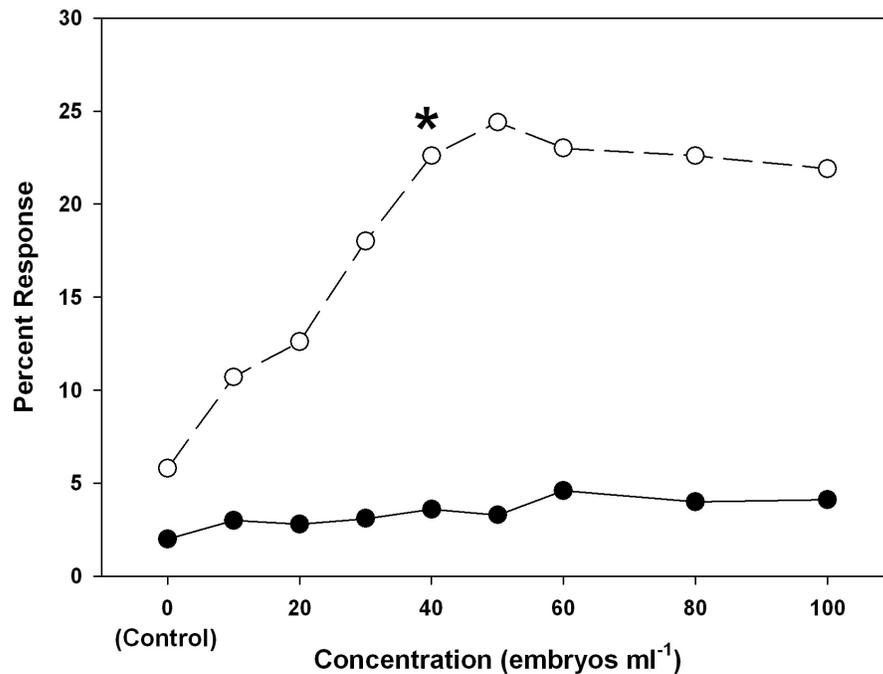


Figure 15: Effect of water containing homogenized early-stage embryos on pleopod pumping response. Percentage of ovigerous *Panulirus argus* with egg masses containing early- (solid line) and late-stage (dashed line) embryos that responded to water containing homogenized early-stage embryos ($n = 15$). Lowest concentration at which pumping response was significantly different from control indicated by an asterisk.

An increase in the concentrations of homogenized late-stage embryo-water (stage VII – VIII) resulted in a significant increase in the responsiveness of females with intact late-stage embryos (Fig.16; $\chi^2 = 23.14$, $df = 8$, $P < 0.005$, $n = 18$), but not for females with early-stage embryos ($P > 0.05$, $n = 18$). Females with late-stage embryos had a response

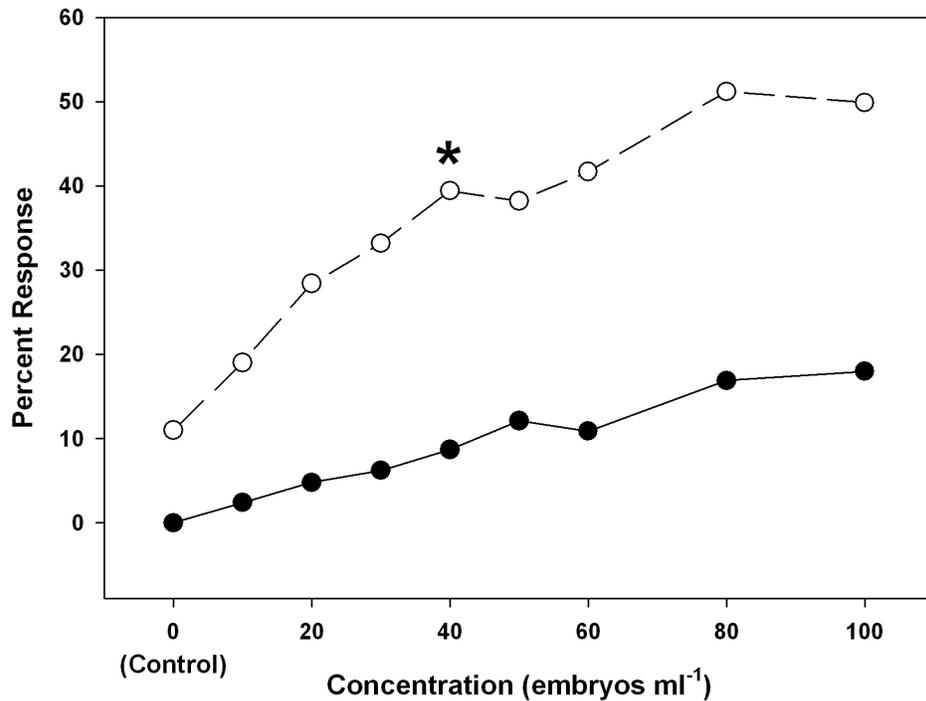


Figure 16: Effect of homogenized late-stage embryos on pleopod pumping response. Percentage ovigerous *Panulirus argus* with egg masses containing early- (solid line) and late-stage (dashed line) embryos that responded to water containing homogenized late-stage embryos ($n = 18$). Lowest concentration at which pumping response was significantly different from control indicated by an asterisk.

threshold of 40 eggs ml⁻¹. The mean percent pumping response (39.4 %) that induced significant responses was almost twice as high, compared to the mean pumping response of late-stage females subjected to homogenized early-stage embryo water (22.6 %, Fig. 3). The lack of a significant response for females with early-stage embryos suggests that only females with late-stage embryos are sensitive to the pheromone. These results also suggest that late-stage embryos contain a higher concentration of the pheromones than early-stage embryos.

3.3.4 Response to Egg-Conditioned Water

Ovigerous lobsters with late-stage embryos responded significantly to water conditioned with late-stage eggs at concentrations above 150 embryos ml⁻¹ (Fig. 17; $\chi^2 = 25.49$, $df = 8$, $P < 0.005$, $n = 18$), but not to water conditioned with early-stage eggs (Fig. 17; $P > 0.05$, $n = 14$). The pumping response increased significantly with increasing

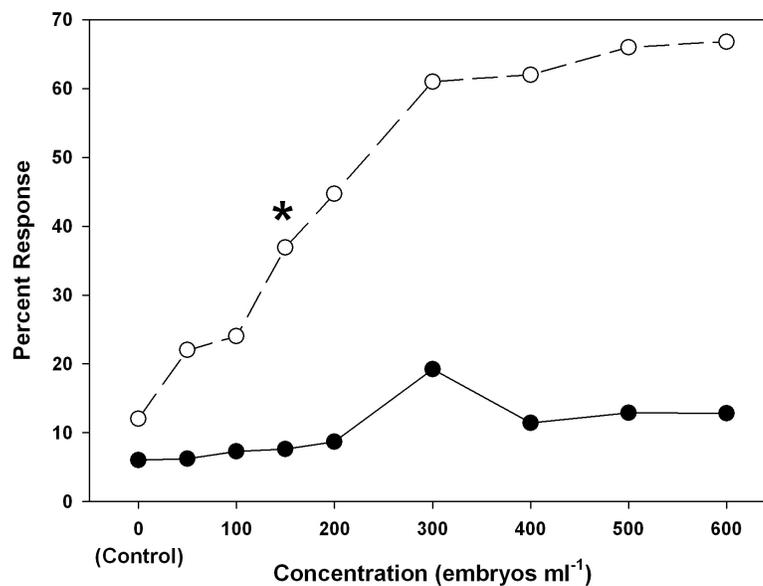


Figure 17: Effect of egg-conditioned water on pleopod pumping response. Percentage of ovigerous *Panulirus argus* with egg masses containing late-stage embryos that responded to water conditioned for 20 h with egg masses containing early- (solid line, $n = 14$) or late-stage (dashed line, $n = 18$) embryos. Lowest concentration at which pumping response is significantly different from controls is indicated by an asterisk.

concentration for water conditioned with late-stage eggs. The concentration necessary to elicit a significant response was 150 eggs ml⁻¹ (Fig. 17). Since both solutions were tested using late-stage females, differences in the dose-response curves suggest that the concentrations of pheromones released from embryos prior to hatching increases as the

embryos mature over time. These results suggest that pheromones are leaching from late-stage embryos prior to hatching, but not from early-stage embryos.

3.3.5 Response to Water Containing Homogenized Egg Cases

Ovigerous lobsters with late-stage embryos responded significantly to water containing crushed post-hatch egg cases (Fig. 18; $\chi^2 = 25.49$, $df = 8$, $P < 0.005$, $n = 12$). The pumping response increased with increasing test concentrations, and the lowest concentration necessary to elicit a significant response was 40 egg cases ml^{-1} . These results indicate that pheromones that stimulate larval release behaviors are associated with the post-hatch egg cases.

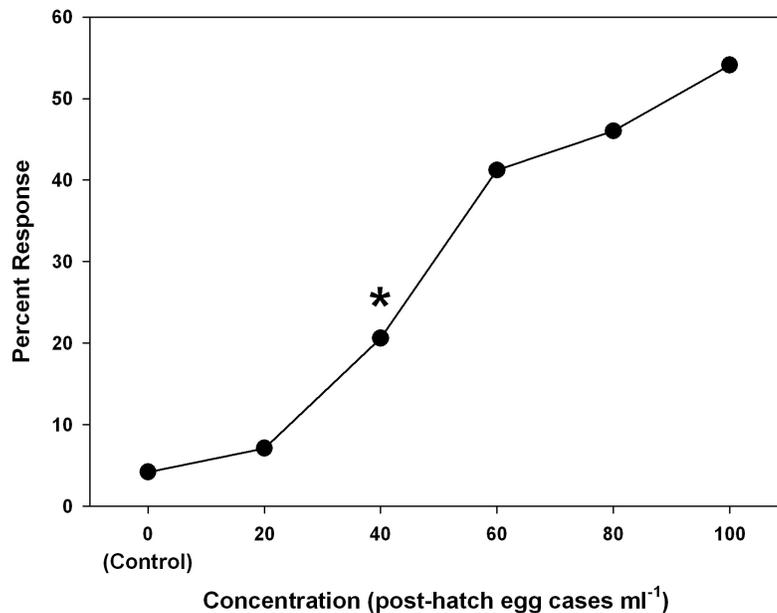


Figure 18: Effect of homogenized post-hatch egg cases on pleopod pumping response. Percentage of ovigerous *Panulirus argus* with late-stage egg masses that responded to water containing homogenized post-hatch embryo cases ($n = 12$). Lowest concentration at which pumping response was significantly different from control was indicated by an asterisk

3.4 Discussion

Substances associated with the hatching embryos evoked the stereotypical larval release behaviors in ovigerous spiny lobster *Panulirus argus*. Ovigerous females placed in water in which larval release had occurred displayed an increase in spontaneous pleopod pumping activity. This result indicates that a chemical cue is involved in the hatching process, and that the pheromones are released at the time of hatching.

Pleopod pumping was observed over the entire duration of embryonic development, however, the level of spontaneous pumping increased dramatically with embryo maturity. These results are similar to those reported for the crabs *Neopanope sayi* (De Vries et al. 1991) and *Callinectes sapidus* (Tankersley et al. 2002). Abdominal pumping by the ovigerous female is a critical maternal care behavior that helps to increase oxygen availability within the egg mass (Baeza and Fernández 2002; Fernández et al. 2002). Abdominal pumping is affected by low oxygen tension inside the egg mass of the crabs *Cancer setosus* and *Homalaspis plana*. As oxygen tension decreased inside the egg mass, an increase in abdominal pumping frequency was detected. In addition, these crabs increased the frequency of abdominal pumping with embryonic development as the oxygen demand of the embryos increased (Baeza and Fernández 2002). This increase in oxygen consumption may be associated with the morphological and functional changes within the embryos (*e.g.*, development of circulatory structures, neuromotor systems, eyes, etc.) which would directly increase metabolism in late-stage embryos.

Therefore, the observed relationship between an increase in spontaneous pleopod pumping activity and embryo maturity for *P. argus* is most likely due to the increased oxygen demand and waste elimination of the embryos (De Vries et al. 1991; Fernández et al. 2002, 2003).

Increased pleopod pumping rates in *P. argus* were induced by larval hatch water. The pumping activity of ovigerous females with both early- and late-stage embryos increased significantly when exposed to increasing concentrations of hatch water, indicating that pheromones stimulating abdominal pumping are released at the time of larval release. The concentrations capable of inducing pleopod pumping behavior were different for lobsters with early- and late-stage embryos, as the threshold concentration was higher for females with early-stage embryos. In addition, overall percent responses were higher for females with late-stage embryos. These results are consistent with studies of the crabs *Rhithropanopeus harrisi* (Forward and Lohmann 1983), *Neopanope sayi* (De Vries and Forward 1991), and *Callinectes sapidus* (Tankersley et al. 2002). Each of these studies found that the pumping response increased as larval hatch water concentration increased. However, crabs with new embryos were less responsive than those with mature embryos (De Vries and Forward 1991; Tankersley et al. 2002).

Saigusa (2000) described embryo hatching for the terrestrial crab *Sesarma haematocheir*, which is highly synchronized with the time of nocturnal high tide (Saigusa 1986a, b, 1992). Ovigerous females subjected to hatch water (filtered medium in which

larvae have been released) liberate their embryos prematurely (Saigusa 1994, 2000), indicating that hatch water contains substances which play a role in hatching. Active factors are released from the embryo itself, and are comprised of two substances: ovigerous hair stripping substance (OHSS) and a caseinolytic protease (Saigusa 1995, 1996, 2000; Ikeda et al. 2006). Experiments with crushed embryos indicated that OHSS appears in embryos about three days prior to hatching (Saigusa 1995). These results suggest that the embryos produce a chemical late in the developmental period that assists with the hatching process. The situation is different for *P. argus*.

The pumping response of ovigerous spiny lobsters subjected to water with homogenized early- and late-stage embryos was variable. Females with early-stage embryos were not significantly responsive to either treatment. However, females with late-stage embryos were responsive at similar threshold concentrations, but the response level was higher upon exposure water with crushed late-stage embryos. This is in contrast to *S. haematocheir*, for which the pumping substance does not appear until three days prior to hatching. Early-stage embryos of *P. argus* contain pheromones that induce larval release behaviors. These results are consistent with *R. harrisii* and *C. sapidus*, which exhibit pumping responses upon exposure to homogenized early- and late-stage embryos (Forward and Lohmann 1983; Tankersley et al. 2002).

The amount of pheromone present within the embryos varied significantly with embryo maturity. Late-stage embryos contained a much larger quantity of the

pheromone than early-stage embryos, as has been reported for *C. sapidus* (Tankersley et al. 2002). These results suggest that there is higher generation of the pheromone as embryo development advances, or perhaps pheromones may accumulate over time within the embryo. This may explain the increase in spontaneous pumping activity with embryo age as more pheromones leach from older embryos. Embryos of several brachyuran crabs including *N. sayi*, *Uca pugilator*, *Sesarma cinereum* (De Vries et al. 1991) and *S. haematocheir* (Saigusa 1992) release enzymes near the time of hatching that aid in the digestion of the embryo membrane. Lobster post-hatch embryo cases are composed of protein (> 70 % for *Homarus gammarus*; Pandian 1970), suggesting that proteolysis of the embryo case would create small peptides (*i.e.*, pumping pheromones) that are detected by the female. At hatching, detection of an increased concentration stimulates the female to increase pleopod pumping behaviors, which accelerates the breakage of embryo cases and facilitate the hatching process. For comparison, abdominal pumping frequencies for the crabs *Cancer setosus* and *Homalaspis plana* were shown to increase when water from late-stage embryos of the crab was pumped into the egg mass of brooding females containing early-stage embryos (Baeza and Fernández 2002). The presence of an unknown compound (*i.e.*, pumping pheromone) could be responsible for the female's behavior.

Water containing crushed post-hatch egg cases induced larval release behaviors in ovigerous spiny lobsters with late-stage embryos. De Vries and Forward (1991) found

that enzymes released by crab embryos cause membrane breakage, and these enzymes work by weakening the inner membrane. These results support the concept that peptide pheromones are released by egg cases and may be generated by enzymes that are acting on the embryo cases, or perhaps, the pheromone is attached to the embryo case during hatching (Rittschof et al. 1990).

Collectively, female spiny lobsters with late-stage embryos are more sensitive to pumping pheromones than females with early-stage embryos. These results indicate that the pheromone increases in concentration as the embryos mature, since the highest concentration of pheromone is found in late-stage embryos. Thus, vigorous pleopod pumping occurs during larval release because female *P. argus* have increased sensitivity to the pheromone, which increases in concentration in mature embryos.

Many of the responses of *P. argus* to these pumping pheromones are consistent with the model for larval release for the subtidal crabs *R. harrisi* (Forward and Lohmann 1983), *N. sayi* (De Vries et al. 1991), and *C. sapidus* (Tankersley et al. 2002). The model hypothesizes that a few embryos hatch spontaneously near the time of larval release, releasing a low concentration of pumping pheromones which, in turn, are detected by the female. In response to this stimulus, the female begins to undergo stereotypical larval release behaviors of increased pleopod pumping. This action physically breaks open more egg membranes, which frees more pheromone. As more pheromone is

released, the female pumps more rapidly. This positive feedback system results in the synchronous release of larvae.

P. argus releases larvae in several batches on consecutive nights at the time of nocturnal slack water after flood tide (Chapter 2). This raises a question as to what cue terminates the pleopod pumping behavior and larval hatching from a female on any given night. Ovigerous females display stereotypical behaviors during larval release that cease after about 20-30 min. It was hypothesized that larval release behaviors cease because the concentration of the pheromone decreases below a threshold concentration as (1) the number of hatching embryos decrease and (2) as the current speed during ebb tide increases thereby transporting the pheromone away from the female. Further research on the model of larval release could determine if this is in fact the case.

By releasing larvae in synchrony at the time of slack water after flood tide, phyllosoma larvae enter the water column at the beginning of ebb tide. This would assist the larvae in being transported seaward by currents to offshore nursery areas for development (Yeung and Lee 2002). Thus, synchronous timing is critical as it ensures that larvae are released at times in which transport is optimal, as well as at times that minimize the threat of visual predation and exposure of larvae to adverse conditions such as low salinity and high temperatures (Forward 1987).

4. Peptide Pheromones and Larval Release Behaviors of the Caribbean Spiny Lobster, *Panulirus argus*

4.1 Introduction

Egg hatching in spiny lobsters, *Panulirus argus*, is a highly synchronous event, occurring before the ebbing tide, which assists the larvae in being transported by currents to offshore nursery areas (Yeung and Lee 2002). This timing is critical as it ensures that larvae are released at times in which transport is optimal, as well as at times that minimize the threat of predation by visual predators and exposure of larvae to adverse conditions (Forward 1987; Morgan 1995).

Synchrony of larval release among marine crustaceans is mediated by chemical-based communication between the hatching eggs and the ovigerous female (Rittschof et al. 1985, 1989, 1990; Forward et al. 1987; De Vries and Forward 1991a, b; De Vries et al. 1991; Rittschof 1993; Chapter 3). Pheromones originating from the hatching embryos induce the ovigerous female to perform stereotypical larval release behaviors (*e.g.*, Ennis 1973, 1975; Branford 1978; Moller and Branford 1979; DeCoursey 1983; De Vries and Forward 1991a; Chapter 3). Among spiny lobsters, during larval release the female lobster uses her walking legs to probe her egg mass while she rapidly flexes and extends her abdomen, all the while actively beating the pleopods back and forth. During larval release, this vigorous 'pleopod pumping' behavior assists in the egg hatching process by compressing the egg mass. Compression of the egg mass helps to break open egg

membranes and free the larvae, which then swim into the water column. As more eggs hatch, more pheromone is released, causing the female to pump harder. This positive feedback system leads to a synchronous release of larvae (Forward 1987; Tankersley et al. 2002; Chapters 2 and 3).

Previous experiments suggest that chemical cues mediate these behaviors among spiny lobsters. Pleopod pumping activity of ovigerous females with late-stage embryos increases when they are exposed to hatch water (the medium into which larvae have been released) (Chapter 3). Solutions of homogenized late-stage embryos and homogenized egg cases also induce larval release behaviors in lobsters with late-stage embryos. These observations suggest that a substance(s) is released at the time of hatching which acts to induce stereotypical pleopod pumping behaviors of the female.

Peptides are effective communication molecules in the marine environment due to their high solubility, high specificity, short half-life, and high signal-to-noise ratio (Rittschof et al. 1992; Rittschof 1993; Pettis et al. 1993; Atema 1995; Decho et al. 1998; Rittschof and Cohen 2004). Small peptides originating from the proteolysis of egg cases may be the compounds responsible for coordinating synchronous release of larvae, since crabs display similar larval release pumping behaviors when exposed to solutions containing specific peptides (Rittschof et al. 1985; Forward et al. 1987; Rittschof et al. 1989). For the crab *Rhithropanopeus harrisi* the developing embryos control the exact time of hatching while the female controls the synchrony of hatching (Forward and

Lohmann 1983). Ovigerous females exposed to partially-identified native peptides or to pheromone analogs initiate the pumping response, a behavior used to measure pheromone activity (Forward et al. 1987; Rittschof et al. 1989; Pettis et al. 1993).

I hypothesize that ovigerous spiny lobsters will respond to the same peptides that produce a larval release pumping response in brachyuran crabs (Forward et al. 1987; Pettis et al. 1993; Rittschof 1993). Measurements of stereotypical larval release behaviors (pleopod pumping frequencies) in response to various model peptides at several concentrations were used to determine which chemical cues are used by lobsters. These substances are collectively termed “pumping pheromones”. I tested a logical series of di- and tripeptides and two oligopeptides: bradykinin, a barnacle settlement pheromone mimic, recently implicated as a larval release pheromone (Rittschof and Cohen 2004); as well as its complement des-Arg⁹-bradykinin, which lacks arginine (Arg) at the basic carboxyl terminus. These compounds were chosen based upon the compositional analysis of partially purified native pheromones from previous studies on brachyuran crabs (*e.g.*, Forward et al. 1987; Rittschof et al. 1985, 1989). No peptides were extracted from the lobsters; rather, I evaluated these logical synthetic candidates for activity. I demonstrate that pleopod pumping responses are evoked by peptides, which have a neutral amino acid at the amino terminus and a basic amino acid at the carboxy terminus, suggesting that specific peptides mimic or act as crustacean pheromones. According to the model for egg hatching, trypsin-like enzymes from hatching eggs

would break down the egg membrane and cleave these peptides that are released in the water. The parent female senses the chemical, which stimulates her to pump her pleopods, and thus results in the synchronous release of larvae.

4.2 Materials and Methods

4.2.1 Collection and Maintenance of Spiny Lobsters

Ovigerous *Panulirus argus* were collected by hand using SCUBA from coral reefs near the Keys Marine Laboratory on Long Key, FL (24° 49.567 N, 80° 48.884 W) from April to August 2006. Lobsters were maintained in individual glass aquaria containing about 75 l of continuously-aerated seawater (salinity of 35-36; 27°C) that was filtered to remove particles > 5 µm. The water in the aquaria was changed daily at random times and the lobsters were fed frozen squid and shrimp every other day. A 14:10 hr photoperiod that simulated the ambient light:dark (LD) cycle at the time of collection was employed.

Prior to experimentation, ovigerous females were classified according to the developmental stage of their egg masses. A small group of embryos ($n = 20$) was removed from the egg mass and examined under a dissecting microscope. Only lobsters with embryos that would hatch within 24–36 hr were tested, since female responsiveness to chemical cues that induce larval release behavior increases with embryo maturity.

Females with late-stage embryos are more responsive than females with early-stage embryos (Chapter 3).

4.2.2 Peptide Solutions

All test chemicals were obtained from Sigma Chemical Company. The peptides used in this study were chosen based on previous studies with brachyuran crabs (*e.g.*, Forward et al. 1987; Rittschof et al. 1989; De Vries and Forward 1991a). These include dipeptides composed of various combinations of neutral, basic, and acidic amino acids (Table 3), as well as several tripeptides and two oligopeptides. According to Rittschof et al. (1985) and De Vries et al. (1991), bioassays using solitary amino acids alone did not induce pumping in previous studies on brachyuran crabs. Therefore, I did not test individual amino acids.

Test solutions containing peptides were prepared immediately prior to use in aged offshore seawater (salinity 35), which had been filtered to remove particles > 0.5 μm . Previous studies found that aged offshore water lacked chemical cues that induce larval release behaviors (*e.g.*, Rittschof et al. 1985, 1989). A stock solution for each peptide was made at 10^{-4} M and diluted for testing. An aliquot of the peptide solution was mixed into 5 l of filtered offshore water to form the appropriate test concentration.

4.2.3 Pumping Assay

The frequency of pleopod pumping upon exposure to the test compounds was used as a bioassay to quantify larval release behavior of the female. The general experimental procedure was, first, to place an ovigerous female in a glass aquarium (50.8 cm x 25.4 cm) containing 5 l of filtered ($> 0.5 \mu\text{m}$) offshore seawater that did not contain the test solution. After a 5-min acclimation period, the number of pleopod movements was counted during a 1-min interval. This count is a measure of the *unstimulated* pumping rate. Second, the same lobster was immediately transferred to an identical aquarium with a test solution and, after 5 min of acclimation, the number of movements in a 1-min period was counted. This second count was the *stimulated* pumping rate. An increase in pleopod pumping rate upon exposure to the test solution was considered a positive response. If pumping activity decreased or stayed the same, the lobster was scored as unresponsive.

For each test solution, a lobster was tested in a series of five or more concentrations, ranging from 10^{-4} to 10^{-12} M. The first solution in the series contained filtered offshore water (concentration = 0 M), which served as a control (the unstimulated pumping rate). Lobsters were tested sequentially with the remaining concentrations proceeding from the lowest to highest, to minimize the chances of sensory adaptation. Individuals were tested once at each test concentration. Each lobster was placed in filtered seawater (control) for at least 30 min before being retested

at the next higher concentration. Preliminary experiments indicated that 30 min was sufficient time between exposures to test solutions for the lobster to return to the same pumping rate as in offshore water (*i.e.*, unstimulated pumping rate). To ensure that test solutions remained active, solutions were remade after 8–10 lobsters were tested.

Pleopod pumping responses to the test peptides were characterized three ways: (1) response threshold concentration, (2) effective concentration range, and (3) maximum percent response (Rittschof et al. 1989). The response threshold concentration is defined as the lowest concentration of a test substance to evoke a significant increase in pumping rate ($P < 0.05$) relative to the pumping rate observed in the control (offshore water). The effective concentration range is the molar concentration range between the response threshold concentrations and the next higher concentration at which the pumping rate returns to the control level. The maximum percent response is the highest percentage of lobsters that responded to a test concentration within the effective concentration range (Forward et al. 1987; Rittschof et al. 1989).

The sample sizes for each test solution ranged from 12 to 20. Responses of lobsters to test solutions were compared to control responses (in filtered offshore water) using a χ^2 -test for multiple proportions and a Dunnett's-type multiple comparisons test (Zar 1999).

4.3 Results

4.3.1 Pleopod Pumping Responses to Dipeptides

Pleopod pumping responses by ovigerous *Panulirus argus* to the test dipeptides varied with the amino acid composition of the peptide (Fig. 19). Pumping responses were elicited by all dipeptides with arginine (Arg) at the carboxy terminus (Table 3).

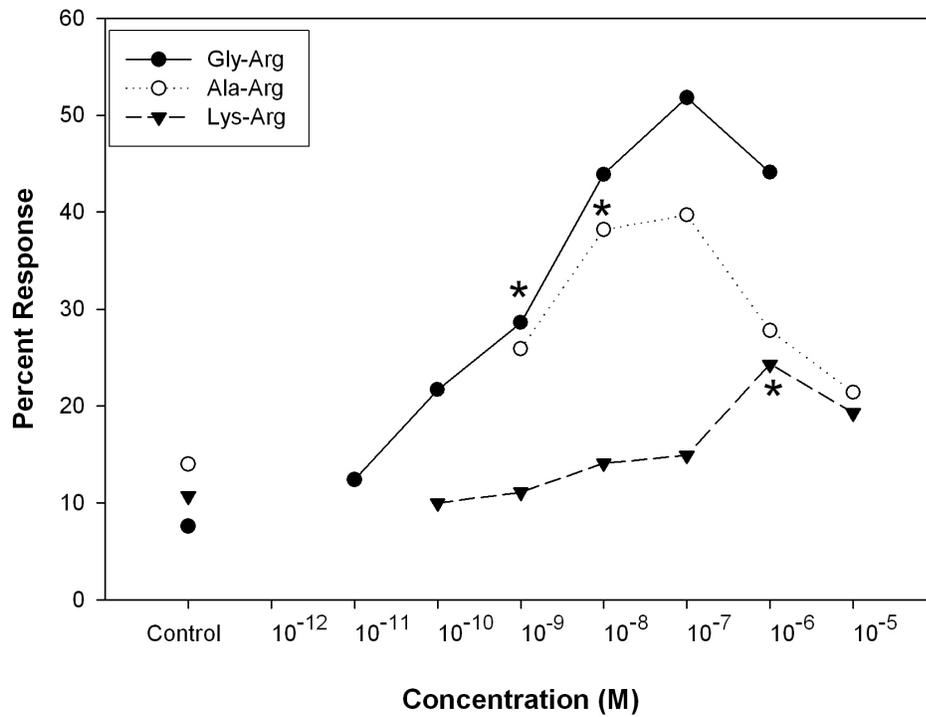


Figure 19: Percentage of ovigerous *Panulirus argus* responding to dipeptides. Control is the pumping response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly greater than the controls ($P < 0.05$) is indicated by an asterisk. ($n = 18$ for all test solutions)

Table 3: Pleopod pumping response measurements of ovigerous *Panulirus argus* to dipeptides.

Dipeptide	Response Threshold Concentration (Molar)	Maximum Response (%)	Effective Concentration Range (log units)
Neutral-Basic			
Gly-Arg	10 ⁻⁹	51.8	4
Ala-Arg	10 ⁻⁸	39.7	3
Basic-Basic			
Lys-Arg	10 ⁻⁶	24.3	2
His-Lys	No response	--	--
Acid-Basic			
Glu-Lys	No response	--	--
Basic-Neutral			
Lys-Gly	No response	--	--
Basic-Acid			
Arg-Glu	No response	--	--
Neutral-Neutral			
Gly-Ser	No response	--	--
Neutral-Acid			
Glu-Asp	No response	--	--
Acid-Neutral			
Glu-Gly	No response	--	--
Acid-Acid			
Glu-Glu	No response	--	--

Response threshold concentrations ranged from 10^{-6} M for Lys-Arg to 10^{-9} M for Gly-Arg. The maximum percent response ranged from 24.3 to 51.8 %, with the greatest number of animals responding to Gly-Arg (Table 3). The effective concentration range for each active dipeptide was at least 2 log units of concentration, and was as high as 4 log units for Gly-Arg. Only dipeptides with a neutral-basic or basic-basic structure were active. No significant pumping responses were observed upon exposure to any of the other types of dipeptides (Table 3).

The dose-response curves for dipeptides that induced a significant response show a similar trend (Fig. 19). As concentration increases, pumping activity rises from a threshold to a maximum level, then declines at higher concentrations, sometimes to a non-significant level (*e.g.*, Ala-Arg).

4.3.2 Pleopod Pumping Responses to Tripeptides

Most of the test tripeptides with Arg at the carboxy terminus produced a significant pleopod pumping response, with the exception of Trp-Ile-Arg (Fig. 20). Response thresholds ranged from 10^{-8} M for Gly-Gly-Arg to 10^{-6} M for Pro-Ile-Arg (Table 4). The maximum response ranged from 38.4 to 58.3 %, with the highest pumping response observed for Gly-Gly-Arg (Table 4). The effective concentration range for each tripeptide was at least 3 log units of concentration.

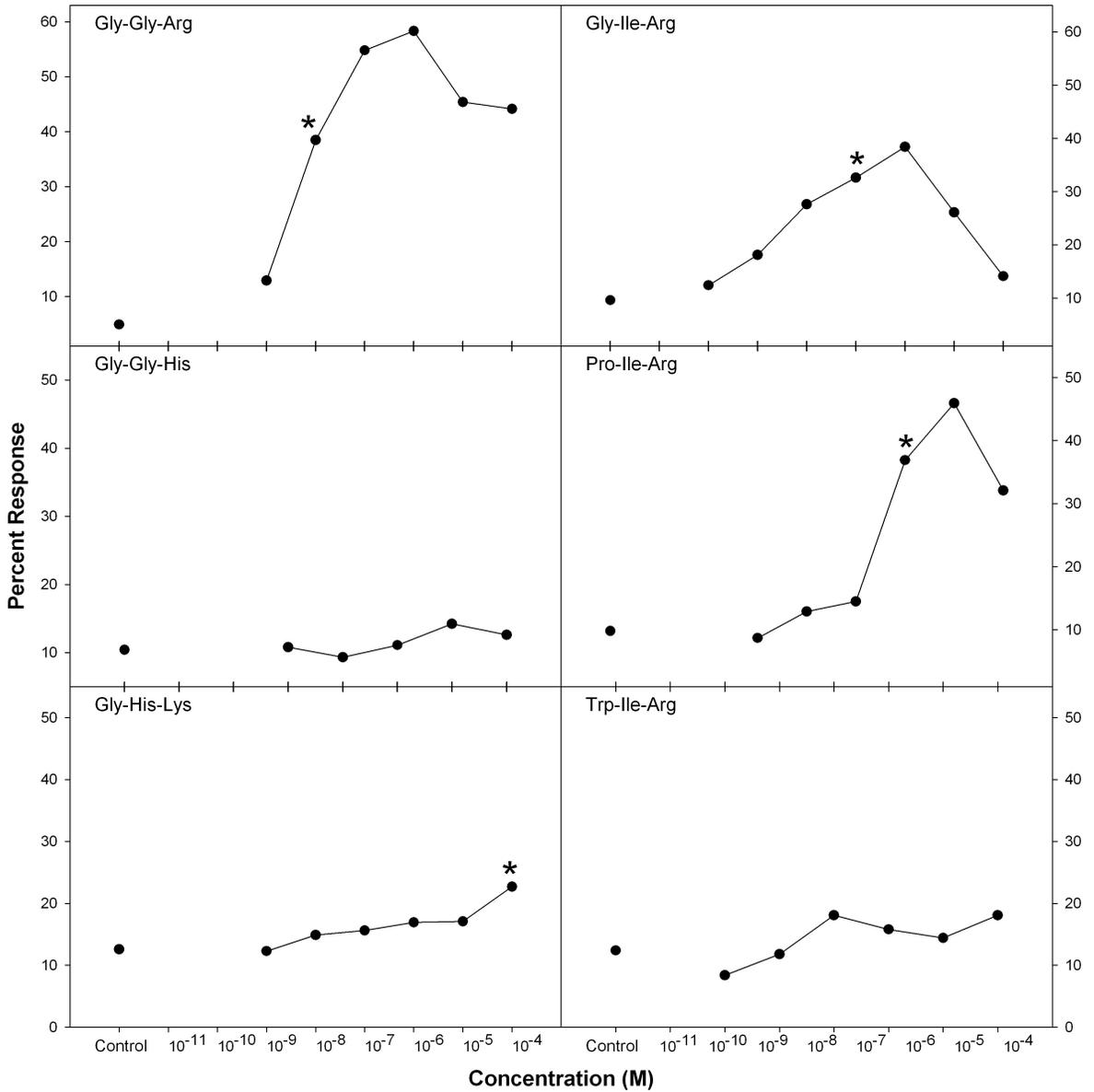


Figure 20: Percentage of ovigerous *Panulirus argus* responding to tripeptides. Control is the response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly greater than the controls ($P < 0.05$) is indicated by an asterisk. ($n = 12$ for Gly-Gly-His and Trp-Ile-Arg, $n = 14$ for all other test solutions)

Table 4: Pleopod pumping response measurements of ovigerous *Panulirus argus* to tripeptides.

Tripeptide	Response Threshold Concentration (Molar)	Maximum Response (%)	Effective Concentration Range (log units)
Neutral-Neutral-Basic			
Gly-Gly-Arg	10 ⁻⁸	58.3	4
Gly-Ile-Arg	10 ⁻⁷	38.4	3
Pro-Ile-Arg	10 ⁻⁶	45.9	3
Trp-Ile-Arg	No response	--	--
Gly-Gly-His	No response	--	--
Neutral-Basic-Basic			
Gly-His-Lys	10 ⁻⁴	26.1	1

The neutral-basic-basic tripeptide Gly-His-Lys also produced a significant pumping response (Fig. 20), with a response threshold of 10⁻⁴ M, and a maximum response of 26.1%, which was the lowest of all tripeptides tested (Table 4). There was no significant pumping response when lobsters were subjected to increasing concentrations of the neutral-neutral-basic tripeptide Gly-Gly-His (Fig. 20; Table 4). These results indicate that neutral-basic amino acid combinations of tripeptides with Arg or Lys at the carboxy terminus are capable of inducing a significant pleopod pumping responses for *P. argus* (Table 4).

The dose-response curves for tripeptides that induced a significant response show a similar trend (Fig. 20). As concentration increases, pumping activity rises from a

threshold to a maximum level, then declines at higher concentrations, but rarely to a non-significant level. An exception is Gly-His-Lys, in which responses modestly increased with increasing concentration. However, the experimental procedure may not have reached the concentration that induces the maximum response.

4.3.3 Pleopod Pumping Responses to Bradykinin

Pleopod pumping rate increased when ovigerous lobsters were subjected to increasing concentrations of bradykinin, an oligopeptide with Arg at the basic carboxy terminus (Fig. 21). Pumping rates were not significantly different from the control when lobsters were subjected to increasing concentrations of des-Arg⁹-bradykinin, an oligopeptide without a basic amino acid at the carboxy terminus (Fig. 21). The response threshold for bradykinin was 10^{-7} M, with a maximum response of 52.9% (Table 5). The effective concentration range for bradykinin was over 3 log units of concentration (Fig. 21; Table 5). These data suggest that the oligopeptide bradykinin has the correct chemical format of neutral-basic amino acid combination at the carboxyl terminal which is capable of inducing a significant pleopod pumping responses for *P. argus* (Table 5).

As the concentration of bradykinin increases, pumping activity rises from a threshold (10^{-7} M) to a maximum level, then declines (Fig. 21). This response pattern was similar to the curves for Gly-Arg, Gly-Gly-Arg, and Pro-Ile-Arg (Figs. 19, 20, 21).

Table 5: Pleopod pumping response measurements of ovigerous *Panulirus argus* to oligopeptide sequences with Bradykinin or Des-Arg⁹-Bradykinin.

Oligopeptide	Response Threshold Concentration (Molar)	Maximum Response (%)	Effective Concentration Range (log units)
Bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	10 ⁻⁷	52.9	4
Des-Arg⁹-Bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	No response	--	--

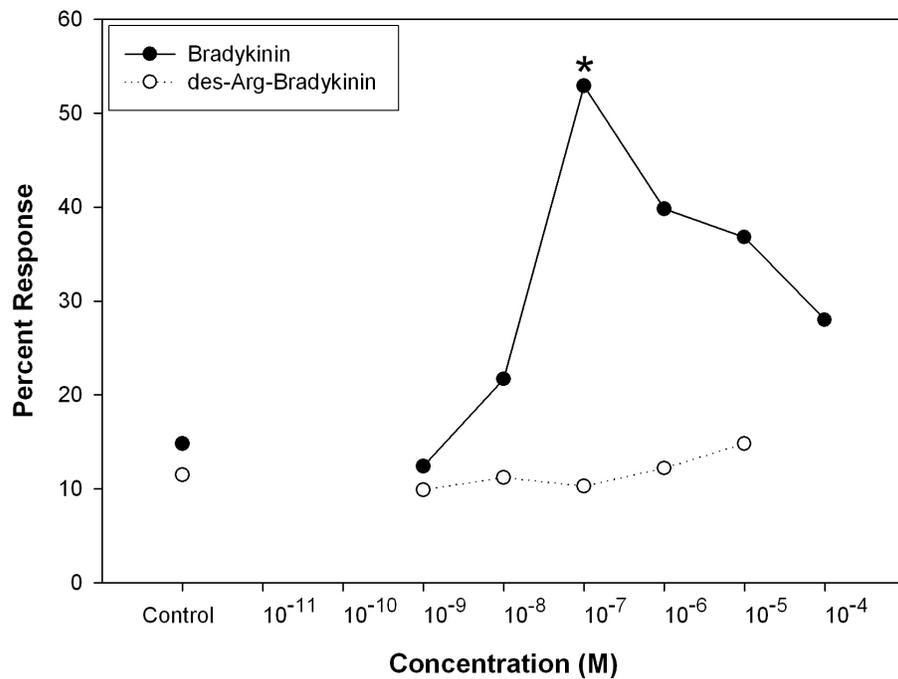


Figure 21: Percentage of ovigerous *Panulirus argus* responding to bradykinin (arginine at the carboxy terminus) or des-Arg-bradykinin (lacking arginine at the carboxy terminus). Control is the response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly greater than the controls ($P < 0.05$) is indicated by an asterisk. ($n = 20$ for both concentrations)

4.4 Discussion

The model for larval release by the spiny lobster *Panulirus argus* proposes that egg hatching readiness is communicated to the female by the release of peptide pheromones from the embryos (Forward and Lohmann 1983; Rittschof et al. 1985; Chapter 3). These pheromones induce the female to undergo stereotypical behaviors involving rapid movements of pleopods. This physical “pumping” activity assists in the rupture of the egg membranes and the subsequent release of free swimming larvae. Peptides which elicit a positive response are considered to be ‘active peptides’ or peptide mimics.

Peptides with Arg or Lys at the carboxy terminus and an adjacent neutral or basic amino acid were capable of inducing a pumping response in ovigerous *P. argus* (Tables 3, 4, 5). The compounds with the lowest threshold concentration for inducing the pumping response were Gly-Arg (threshold at 10^{-9} M), followed by Gly-Gly-Arg and Ala-Arg (thresholds both at 10^{-8} M). Bradykinin was able to induce pleopod pumping in *P. argus* at a response threshold of 10^{-7} M. This result suggests that increased potency might be achieved by decreasing the length of the peptide, the shortest peptide (Gly-Arg) had the lowest threshold concentration.

In terms of response threshold, peptides with Arg at the carboxy terminus are more active than those containing Lys (Tables 3, 4). Arg has an additional amino group, and thus, is more basic than Lys. In addition, Lys has a shorter chain length (R group)

with a lower pK_a , therefore these structural differences may explain the differences in response levels. The basic tripeptide Gly-His-Lys produced a significant pumping response (Fig. 20); however, the concentrations necessary to produce a significant response were $> 10^{-4}$ M. This is quite high in comparison to peptides with Arg. Active peptides containing Lys at the carboxy terminus were able to induce a significant response in the mud crab *Rhithropanopeus harrisi*, but only at threshold levels that were also higher than amounts possible in the natural pumping pheromones (Rittschof et al. 1985; Forward et al. 1987). Thus, peptides containing Lys at the carboxy terminus may not be used as pheromones by *P. argus*.

The peptides tested that induced significant responses were at similar concentrations to the concentrations predicted for the native pheromones in brachyuran crabs (Forward et al. 1987; Rittschof et al. 1989). The lower threshold for the most active peptides tested (Gly-Arg, Gly-Gly-Arg, Ala-Arg, and Bradykinin) ranged from 10^{-7} to 10^{-9} M for *P. argus*. These thresholds are 3–4 orders of magnitude below those peptides used as pheromones by other marine invertebrates, such as the sand dollar *Dendraster excentricus* (Burke 1984), the abalone *Haliotis* (Morse 1988), the barnacle *Balanus amphitrite* (now known as *Amphibalanus amphitrite*) (Tegtmeyer and Rittschof 1989), and the cnidarian *Cassiopeia andromeda* (Fleck 1998; Walther and Fleck 1998). Therefore, sufficient amounts of these peptides could be released by the eggs to serve as pheromones.

The chemical induction of the behavioral response occurs only within a narrowly defined concentration range. Responses typically decline when the concentrations are either more or less than one log unit from the concentration that evokes the greatest responses (Forward et al. 1987; Rittschof et al. 1989; Browne et al. 1998; Decho et al. 1998). Peptides, such as Ala-Arg, Gly-Ile-Arg, and bradykinin, are inhibitory at high concentrations (Derby and Atema 1982, 1988; Rittschof et al. 1989).

These results are consistent with previous studies of chemically induced larval release in crustaceans. The chemical nature of pumping pheromones for brachyuran crabs has been determined to be a heterogeneous group of small peptides containing Arg (Rittschof et al. 1985, 1989; Forward et al. 1987). Behaviorally active peptides have a general structure of a neutral amino acid at the amino terminus (*e.g.*, alanine) coupled to a basic amino acid at the carboxy terminus (*e.g.*, arginine) (Forward et al. 1987; Rittschof et al. 1989; Pettis et al. 1993). Other common characteristics of these active peptides include a positively charged R group attached to the carboxy-terminal and a hydrophobic R group attached to the amino-terminal end of the molecule (Pettis et al. 1993).

Peptides capable of inducing the pumping response in the crab *Rhithropanopeus harrisi* have Arg as the carboxy terminal residue and a neutral amino acid at the hydrophobic residue (Forward et al. 1987). An amino acid analysis of the pumping factors indicated that histidine (His) was not among the amino acids associated with *R.*

harrissi (Rittschof et al. 1985), even though Gly-His-Lys produced a significant pumping response (Forward et al. 1987). This is consistent with our results for *P. argus*, in which the tripeptide Gly-His-Lys produced a significant response while Gly-Gly-His did not induce a pumping response. These results suggest that His cannot be the carboxy terminal residue in the peptide pheromone.

Although His is a basic amino acid, it contains an imidazole ring structure, and thus, may lack the necessary components to act as a chemical signal in this system. Lys and Arg are more basic amino acids than His. The pK_a values for Lys and Arg indicate that their side chains are always positively charged under physiological conditions. These amino acids are strongly polar and as a consequence can easily be hydrated by the surrounding aqueous environment. When His is incorporated into proteins, the pK_a is raised to about 7. The imidazole can exchange protons at physiological (neutral) pH, and thus, it often plays a role in enzymatic catalysis (Mathews et al. 1999).

Peptides with Arg or Lys at the carboxyl terminus are produced by trypsin-like serine proteases, which cleave after basic amino acids. Therefore, the pheromones that induce pumping behavior may be generated by trypsin-like serine proteases capable of digesting the membrane around the embryos of spiny lobsters. Trypsin-like activity may result from enzymes released by embryos (Rittschof et al. 1990; De Vries and Forward 1991b). The brachyuran crabs *Neopanopae sayi*, *Uca pugilator*, and *Sesarma cinereum* release proteolytic enzymes near the time of hatching (De Vries and Forward

1991b). These enzymes assist with the breakage of the outer membrane of the egg case, resulting in the emergence of a free swimming larva. A logical extension of this work is to test the hypothesis that peptide pumping pheromones are created by proteolytic activity (Rittschof et al. 1990; see Chapter 5).

Results from the present study support the hypothesis that larval release behaviors are induced by active substances (pheromones) composed of small peptides. The active peptides have a specific amino acid sequence of neutral-basic and neutral-neutral-basic di-, tri- and oligopeptides. These biologically potent peptides are effective at concentrations from 10^{-4} to 10^{-9} , which is in agreement with previously published studies on brachyuran crabs (Rittschof et al. 1985; Forward et al. 1987; Rittschof and Cohen 2004). The results of this study support the conceptual model that larval release in spiny lobster *P. argus* is controlled by pheromones released from hatching eggs, which acts to induce the stereotypical pumping behaviors of the female that synchronizes larval release.

Releasing larvae in synchrony with the time of slack water before the ebbing tide allows the phyllosoma larvae to enter the water column at a beneficial time for transport by currents to offshore nursery areas for development (Yeung and Lee 2002). This synchronous timing also ensures that larvae are released at times that minimize the threat of visual predation (at night) and exposure of larvae to adverse conditions such as low salinity and high temperatures (Forward 1987).

5. Larval Release Behaviors in the Caribbean Spiny Lobster, *Panulirus argus*: Role of Trypsin-Like Serine Proteases

5.1 Introduction

Larval release in the Caribbean spiny lobster *Panulirus argus* is highly synchronous, with hatching occurring at the time of slack water after nocturnal flood tide (Chapter 2). I have developed a model to describe this precise hatching process. Spiny lobsters undergo stereotypical behaviors, in which lobsters actively flex and extend their abdomens, while rhythmically beating their pleopods to aid in the egg hatching process (Ennis 1973, 1975; Chapters 2 and 3). Peptide pheromones (pumping factors, Rittschof et al. 1985; 1989) are used as a communication signal from the embryos to the female, causing her to perform the larval release behaviors. These behaviors help to break open egg membranes and free the larvae. Since synthetic peptides that contain arginine or lysine at the carboxyl terminal with at least one neutral amino acid induces larval release behaviors in *P. argus*, the larval release pheromones are likely to be oligopeptides with this carboxyl terminal arrangement (Chapter 4).

The current model for larval release among decapod crustaceans hypothesizes that enzymes released by the embryos lead to the enzymatic digestion of egg membranes, and thus produce pumping factors in preparation for hatching (DeVries and Forward 1991a, b; De Vries et al. 1991). Evidence for enzymatic control comes from previous studies on brachyuran crabs. Proteolytic enzymes are released from the

embryos of the crab *Sesarma cinereum*, *Uca pugilator*, and *Neopanopeus sayi* near the time of egg hatching (De Vries and Forward 1991a, b). Results of casein assays indicated that embryos release enzymes at the same time that the egg membrane is broken (De Vries and Forward 1991a, b). Egg membranes on the embryos on *P. argus* begin to break open approximately 15 min prior to larval release and the integrity of the egg membrane changes from a tough cuticle to a more smooth and flexible structure (Ziegler, personal observation). This sequence is in contrast to the subtidal crab *N. sayi*, in which egg membrane breakage occurs several hours before the time of larval release by the female. *N. sayi* also has a low level of enzyme activity several hours preceding the time of larval release (De Vries and Forward 1991a).

Based on these observations, I hypothesized that at the time of hatching by *P. argus*, peptide pheromones are generated by the proteolytic digestion of proteins within the egg membranes by trypsin-like enzymes (Rittschof et al. 1990). To test this hypothesis, I first determined if peptide pheromones could be generated by digestion of egg membrane proteins by trypsin. Trypsin is a serine protease that cleaves peptide bonds in proteins, after basic amino acids. The peptides generated from this process would have an arginine (Arg) or lysine (Lys) at the carboxyl terminal. Trypsin-like activity may occur from enzymes released by bacteria, embryos, or the female herself and could be responsible for generating active peptides.

Peptides triggering larval release behaviors bind to specific receptors thereby initiating a cascade of events leading to the transduction of a chemical signal (Mathews et al. 1999). To indirectly investigate the peptide receptor site, we studied the effects of a trypsin inhibitor on the female's larval release behavior, since trypsin inhibitors are known to mimic pumping factors (Rittschof et al. 1990). Trypsin inhibitors are examples of folded and constrained proteins which forms a 1:1 complex with trypsin with a high affinity and specificity. The active site of trypsin binds to a specific portion of the inhibitor's protein, while the other parts of the tertiary structure of the molecule add to the high affinity of binding. Trypsin inhibitors render the trypsin enzyme inactive when they bind to its catalytic site (Rittschof 1980b; Rittschof et al. 1990). Thus, I tested a second hypothesis, that trypsin inhibitors are capable of mimicking the peptide pheromones capable of inducing the larval release behaviors in *P. argus*. If pleopod pumping behaviors are stimulated by trypsin inhibitors in *P. argus*, then the peptide pheromone receptor site and the trypsin catalytic site may be similar in structure.

5.2 Materials and Methods

5.2.1 Collection and Maintenance of Spiny Lobsters

Ovigerous *Panulirus argus* were collected by hand on SCUBA from coral reefs near the Keys Marine Laboratory on Long Key FL (24° 49.567 N, 80° 48.884 W) during the summer of 2006. Following collection, lobsters were placed in the laboratory in

individual glass aquaria (50.8 cm x 25.4 cm) filled with aged offshore water (water collected a minimum of 3 weeks prior to use) that was filtered to remove particles > 0.5 μm . Lobsters were maintained under a 14:10 h photoperiod that simulated the ambient light:dark (LD) cycle at a constant temperature of $27 \pm 0.5^\circ\text{C}$. Lobsters were held under these conditions for approximately 24-48 h prior to experiments.

5.2.2 Experimental Procedures

I tested the effects of trypsin on premature egg hatching. The time of egg hatching is predictable by examining the embryos microscopically (Chapter 2). Late-stage embryos are easily identified as the egg mass was dark brown in color, and the eyespot of the developing larva was visible to the naked eye. When the embryo eyespot is oval or round in shape, hatching will occur within 36 hr. Only lobsters that were 36 hr from hatching were used in these experiments. All lobsters had a similar carapace length (~ 69-76 mm CL).

As a control, ovigerous lobsters ($n = 15$) were incubated in filtered seawater and the number of detached embryos and prematurely-hatched larvae were recorded. For the experimental treatment, ovigerous females with late-stage embryos ($n = 10$) were placed in a glass aquaria containing 5 l of a test concentration of trypsin (in a range from 10^{-9} to 10^{-4} M) for an incubation time of 4 hr. Incubation in trypsin was conducted at times during the LD cycle in which lobsters rarely release larvae (the middle of the light phase, Chapter 2). At the end of the incubation period, the lobster was removed from

the aquarium and the water was filtered to obtain all detached embryos and larvae. The number of embryos that became detached from the female (eggs) and the embryos that hatched prematurely (larvae) were then counted. Premature egg hatching does not normally occur for *P. argus* (Ziegler, personal observation), therefore, any detached embryos or premature larvae observed were ascribed to trypsin activity.

5.2.3 Preparation of Test Solutions

All test chemicals were purchased from Sigma Chemical Company. I used trypsin (from porcine pancreas, Type II-S Sigma # T7409-1G; 10,000 BAEE units mg⁻¹) or trypsin inhibitor (from turkey egg-white, Type II-T, Sigma # T4385; 10,000 BAEE units mg⁻¹) for these experiments, based on previous results on brachyuran crabs (Rittschof et al. 1990; De Vries and Forward 1991). Trypsin and trypsin inhibitor test solutions were prepared immediately prior to use. Test solutions were prepared at a concentration of 1 mg/ml of compound in deionized water. Trypsin and trypsin inhibitors were combined at a 1:1 ratio. Stock solutions were then diluted in 1000-fold increments. An aliquot of the test solution was then pipetted directly into the seawater in the test aquarium, with final concentrations being 10⁻⁹ to 10⁻⁵ M for trypsin, 10⁻⁹ to 10⁻⁵ M for trypsin inhibitor alone, and 10⁻¹¹ to 10⁻⁴ M for trypsin and trypsin inhibitors combined.

Trypsin solutions were made with the following reagents recommended by Sigma Chemical Company. Trypsin was dissolved in 1 mM hydrochloric acid in 50 ml of deionized water solution 15 min prior to use. Immediately prior to the experiment, I

added the trypsin solution to 100 ml of sodium phosphate buffer (Sigma #S0751) at a pH of 7.8 at 27°C.

5.2.4 Pumping Activity Bioassay

I used pleopod pumping behavior as a bioassay to determine the effects of trypsin and a trypsin inhibitor, as well as the combined effects of both trypsin and trypsin inhibitor (Forward et al. 1987; Rittschof et al. 1990) on the induction of the females' larval release behavior. Bioassays were conducted under dim red light in the laboratory at random times between the 10:00 and 20:00 h to control for any possible rhythm in pumping activity. The assay consisted of placing an ovigerous lobster in a glass aquarium (50.8 cm x 25.4 cm) containing 5 l of aged, filtered (> 0.5 µm) offshore water. The number of pleopod movements during a 1 min period was recorded as the control pumping rate. The lobster was then transferred to an identical aquarium containing a test solution (5 l), and the number of pleopod movements over a 1 min interval was counted after a 5 min acclimation period. An increase in pleopod pumping rate upon exposure to the test solution was considered a positive response. If pumping activity decreased or stayed the same, then the lobster was scored as unresponsive. The percent of lobsters that responded positively served as a relative measure of the biological activity of the test solution.

To minimize the chance of sensory adaptation, the test concentrations proceeded from the lowest to highest. Individuals were tested once at each test concentration.

Each lobster was placed in filtered seawater (control) for at least 30 min before being retested at the next higher concentration. Preliminary experiments indicated that 30 min was sufficient time between exposures to test solutions for the lobster to return to the same pumping rate in offshore water (control pumping rate). To ensure that test solutions remained active, solutions were remade after every 6 or 7 lobsters tested.

Pleopod pumping responses to all test compounds were described as: (1) response threshold concentration, (2) effective concentration range, and (3) maximum percent response (Rittschof et al. 1989, 1990). The response threshold concentration is the lowest concentration to induce a significant increase in pumping rate ($P < 0.05$) relative to the control (filtered offshore water). The effective concentration range is the molar concentration range over which the test compound induce a significant pumping response relative to the control. The maximum percent response is the highest percentage of lobsters responding to the test compound within the effective concentration range. Pumping rate responses of ovigerous lobsters to the test solutions were compared to the pumping rate of lobsters in offshore water alone (control) using a χ^2 -test for multiple proportions and a Dunnett's-type multiple comparison test (Zar 1999).

5.2.5 Effect of Trypsin on Hatch Water

Larval hatching occurs at a specific time and is highly synchronized with the time of slack water after flood tides (Chapter 2). Hatch water was prepared by placing

an ovigerous lobster in an aquarium (100.5 cm x 30.6 cm) containing filtered seawater (0.5 μm , salinity ≈ 35). Immediately following the hatching event, the female was removed from the aquarium. Hatch water was then passed through a 5 μm filter which removed the larvae, egg membranes, and any unhatched embryos. The titer of the pheromones in the hatch water was estimated by counting the number of larvae in 10 subsamples of hatch water. The pheromone concentration of the hatch water was expressed as larvae ml^{-1} . A uniform amount of pheromone was assumed to be released upon hatching of each embryo. Hatch water was used within 2-4 h after hatching, and diluted with filtered offshore water to produce the test concentration of 100 larvae ml^{-1} . This concentration was selected for its consistency to provide a pumping response (Chapter 3).

This experiment was divided into three treatments ($n = 12$ for each treatment): one group of animals was treated to offshore water alone, a second group was subjected to a treatment of hatch water at a concentration of 100 larvae ml^{-1} , while a third group was subjected to a solution of hatch water (100 larvae ml^{-1}) that was treated with trypsin. For the third treatment, 5 mg of porcine trypsin (50,000 BAEE units) was dissolved into 2 l of hatch water immediately prior to use. Ovigerous females with late-stage embryos were then tested in the hatch water-trypsin solution. The number of pleopods movements per min was counted after a 10-min acclimation period. After 4 h, the number of detached embryos and prematurely hatched larvae were counted in each

treatment. Preliminary experiments indicated that the pheromone in hatch water is still viable and able to stimulate pumping for over 24 h after a hatching event. The responses of lobsters to test solutions were compared to control responses (in filtered offshore water) using a χ^2 -test for multiple proportions and a Dunnett's-type multiple comparisons test (Zar 1999).

5.3 Results

5.3.1 Effect of Trypsin on the Egg Mass

The premature release of eggs and larvae was observed after exposing ovigerous *P. argus* to exogenous porcine trypsin for 4 h (Fig. 22). No larvae or eggs were released in offshore water alone. Premature egg and larval release increased with increasing concentrations of trypsin. The dose-response curve tends to level off at higher concentrations, suggesting a saturation effect (Fig. 22). The lowest effective concentration of trypsin that caused a release of eggs 10^{-7} M (Fig. 22; $\chi^2 = 11.07$, $df = 5$, $P < 0.05$, $n = 10$), or caused the release of larvae was 10^{-8} M (Fig. 22; $\chi^2 = 16.75$, $df = 5$, $P < 0.005$, $n = 10$).

Female lobsters responded behaviorally to the trypsin solution by moving their walking legs in an agitated manner. The mechanical actions of the female may have assisted in the release of eggs and larvae. This behavior was not observed in offshore

water alone. The premature detachment of fertilized eggs is uncommon in the laboratory setting, although females are capable of grooming their egg mass and

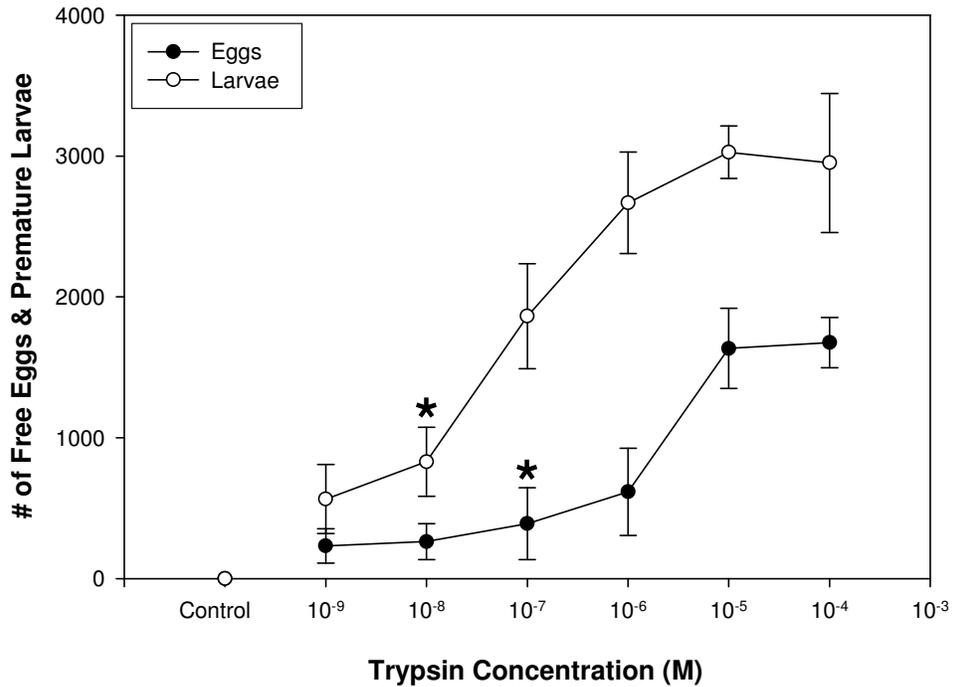


Figure 22: Number of prematurely released eggs and larvae after incubation of ovigerous *Panulirus argus* to different concentrations of porcine trypsin. Control indicates the number of free larvae and eggs found for a female exposed to filtered offshore water alone. Means and SE are plotted. ($n = 10$)

removing non-fertilized eggs (Ziegler, personal observation). Larvae that hatched prematurely did not swim, although, they were alive. Microscopic inspection of the larvae revealed a heartbeat and slight movements of the appendages. This suggests that premature hatching was a result of the enzymatic process rather than by embryo actions.

5.3.2 Effect of Trypsin on Pleopod Pumping Response

Ovigerous lobsters with late-stage embryos responded to trypsin (Fig. 23; $\chi^2 = 16.75$, $df = 5$, $P < 0.005$, $n = 15$). The pumping response increased significantly with increasing concentration of trypsin to a response threshold level of 10^{-7} M, and then declined slightly at higher concentrations. The maximum percent response was 48.2 % (Table 6). The effective concentration range for trypsin was at least 3 log units of concentration.

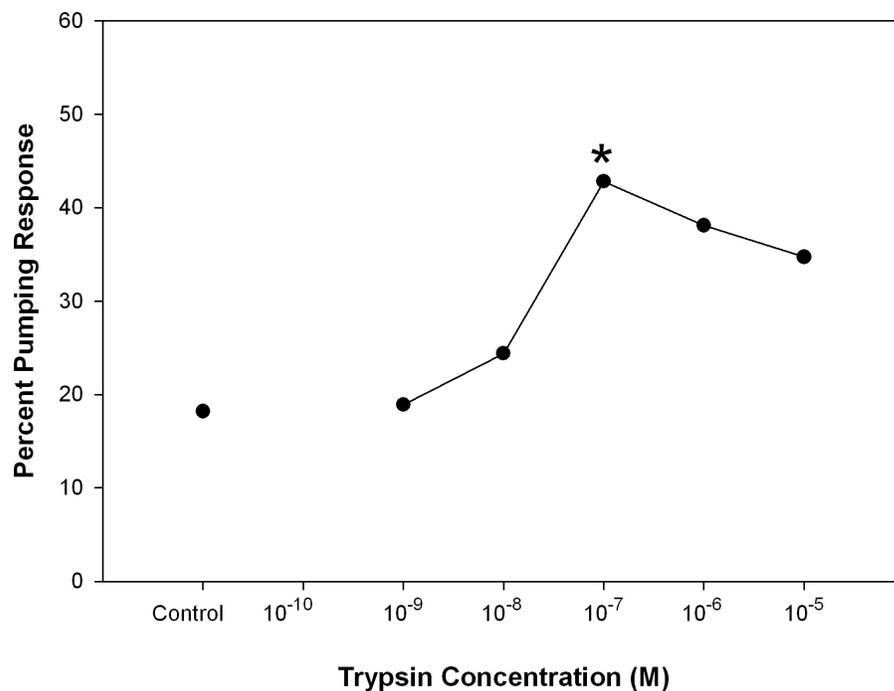


Figure 23: Percentage of ovigerous *Panulirus argus* that increased pleopod pumping rate upon exposure to different concentrations of porcine trypsin and offshore water. Control is the response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly different from control ($P < 0.05$) is indicated by an asterisk. ($n = 15$)

5.3.3 Effect of Trypsin Inhibitors on Pleopod Pumping Response

Ovigerous lobsters with late-stage embryos responded to trypsin inhibitor (Fig. 24; $\chi^2 = 16.75$, $df = 5$, $P < 0.005$, $n = 15$). The pumping response increased significantly with increasing concentration of trypsin inhibitor, however, the response leveled off at higher concentrations (Fig. 24). The lowest concentration necessary to elicit a

Table 6: Pleopod pumping response measurements of ovigerous *Panulirus argus* to test chemicals. ($n = 15$ for all treatments)

Test Chemical	Response Threshold Concentration (Molar)	Maximum Response (%)	Effective Concentration Range (log units)
Trypsin	10^{-7}	42.8	3
Trypsin Inhibitor	10^{-9}	51.3	5
Trypsin + Trypsin Inhibitor	No response	--	--

significant response was 10^{-9} M (Table 6), and the maximum pumping response occurred at 10^{-8} M. The maximum percent response was 51.3 %, while the effective concentration range for trypsin was at least 5 log units of concentration (Table 6).

5.3.4 Effect of Trypsin Combined with Trypsin Inhibitor on the Pumping Response

The pleopod pumping response was eliminated when ovigerous females were subjected to the combination of both trypsin inhibitor and porcine trypsin (Fig. 25; $P > 0.05$, $n = 15$).

This combination appears to be inhibitory at concentrations of 10^{-8} to 10^{-7} M, although the percent response at each concentration was not significantly lower than the control. This result indicates that the trypsin-trypsin inhibitor complex is inactive as a pheromone mimic.

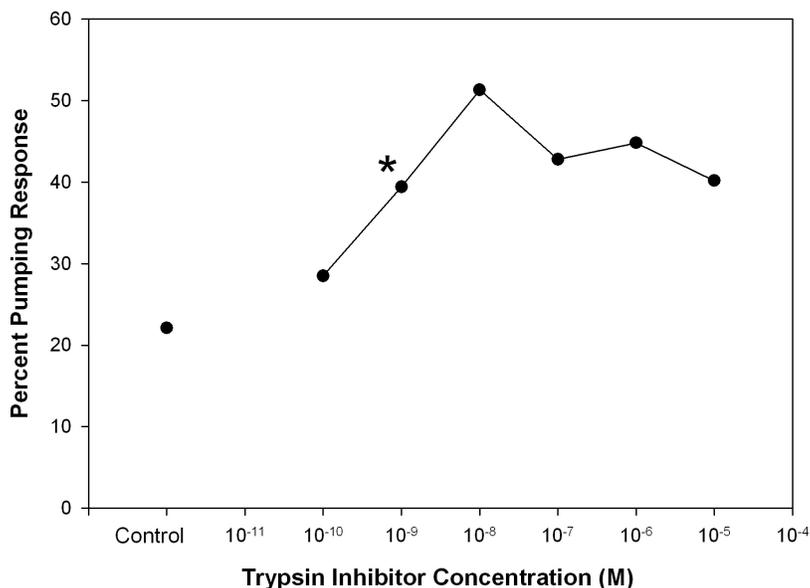


Figure 24: Percentage of ovigerous *Panulirus argus* that increased pleopod pumping rate upon exposure to different concentrations of trypsin inhibitor combined with offshore water. Control is the response level upon exposure to filtered offshore water alone. Lowest concentration at which the pumping response was significantly different from controls ($P < 0.05$) is indicated by an asterisk. ($n = 15$)

5.3.1 Effect of Trypsin on Hatch Water

Increased pleopod pumping activity ceased upon exposure to hatch water that was treated with trypsin (Table 7). The addition of trypsin to hatch water for a 4 hr period created a large amount of peptides (estimated to be over 50,000 BAEE units/min).

This large amount of peptides present in the solution most likely caused a saturation effect which overwhelmed the pheromone receptors, and thus terminated the larval release behaviors of the female.

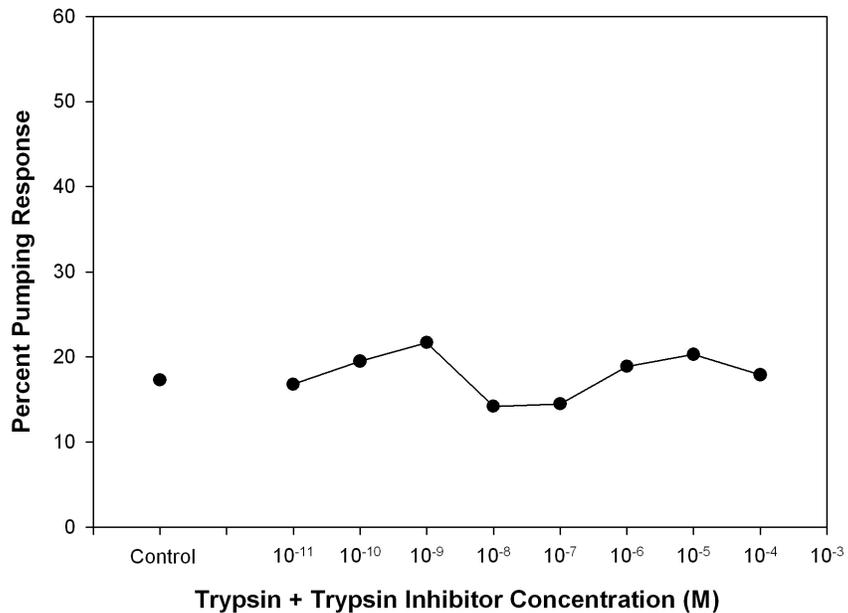


Figure 25: Percentage of ovigerous *Panulirus argus* that increased pleopod pumping rate upon exposure to different concentrations of both trypsin and trypsin inhibitors combined. Control is the response level upon sequential exposure to filtered offshore water. In no case is the percent response significantly higher from the controls. ($n = 15$)

Table 7: Effect of trypsin on hatch water (100 larvae ml⁻¹) of ovigerous *Panulirus argus*. ($n = 12$ for all treatments)

Treatment	Mean Pumping Response (% \pm SD)
Filtered seawater	26.5 % (± 1.6)
Hatch water	69.1 % (± 3.5)
Trypsin + Hatch water	12.7 % (± 2.8)

5.4 Discussion

I hypothesized that peptide pheromones could be generated by the proteolytic cleavage of egg membrane proteins. When ovigerous spiny lobsters were exposed to different concentrations of porcine trypsin, I observed the detachment of eggs and premature hatching of larvae. This result suggests that the enzyme is capable of breaking down egg membranes. These results are similar to a study on the mud crab *Rhithropanopeus harrisi* (Rittschof et al. 1990). Embryos of the mud crab detached upon exposure to either bovine or porcine trypsin and were released into the medium by the pumping movements of the female. Some of the embryos prematurely hatch as immobile larvae (Rittschof et al. 1990). De Vries and Forward (1991a) reported that a proteolytic enzyme is released from the embryos near the time of egg hatching in several species of estuarine crabs.

Upon exposure to exogenous trypsin, ovigerous lobsters responded with increased pumping rates, indicating that the enzyme is breaking down proteins associated with the eggs to produce peptide pheromones. These pheromone signals were received by the female lobster, and she responded by increasing the rate of movement of her pleopods. Results of chapter 4 demonstrated that the pumping response declines upon exposure to high concentration of peptides that mimic the pheromones. These results support the concept that trypsin-like enzymes are

responsible for the degradation of the egg membrane at the time of hatching to produce peptide pheromones (Pettis et al. 1993; Rittschof 1993).

When trypsin was added to hatch water of *P. argus*, additional pumping pheromones were produced by trypsin, and pumping ceased. One explanation for this result is that an enormous amount of peptides were present in the solution, causing a saturation effect which terminated the larval release behaviors of the female. Additional trypsin was also generating peptide pheromones from the hatch water, in addition to those already present in the water. Pumping pheromones made of peptides are common among decapod crustaceans and inhibition of their responses at high concentrations is well known (Rittschof 1993).

Although trypsin seems to be coming from the embryos, I also observed a possible situation where an enzyme may be released from the female as well. Observations of the female abdomen of *P. argus* just after larval release indicate that the broken egg case and funiculus remain attached to the ovigerous hairs. A similar event occurs with the lobsters *Homarus americanus* and *H. gammarus* in which egg cases and stalks remain attached on the ovigerous hairs and are not cast off until the next molt (Talbot and Harper 1984; Goudeau et al. 1987). For *P. argus*, however, the egg cases are removed from the ovigerous hairs less than 24 h after the time of larval release (Ziegler, personal observation). The substance that assists with the breakage of the egg cases may

also be the same enzyme that assists with the removal of egg cases after the hatching event.

This study indirectly investigated the nature of the pheromone receptor by determining whether the female's larval release behavior could be generated by exposure to a trypsin inhibitor. Trypsin inhibitors, in general, have the ability to bind to the catalytic site of trypsin. Larval release pumping behaviors were induced in ovigerous females subjected to increasing concentrations of trypsin inhibitors. Pumping responses evoked by trypsin inhibitors had a low threshold concentration (10^{-9} M). The concentrations capable of inducing a response are in the same range of the active peptides tested (Chapter 3) and are also similar to the range of concentrations of native pheromone for the crab *Rhithropanopeus harrisi* (Rittschof et al. 1985; Forward et al. 1987). An increase in pumping rates was also observed for ovigerous females of the crab *R. harrisi* that were subjected to increasing concentrations of trypsin inhibitors (Rittschof et al. 1985; Forward et al. 1987). Thus, trypsin inhibitors were acting as a pheromone mimic, causing the pleopod pumping response to increase with increasing concentration.

When trypsin inhibitors were combined with trypsin, larval release behaviors ceased to occur. This result suggests that interaction with the trypsin catalytic site is necessary to produce peptides capable of inducing the pumping response. Trypsin inhibitor works as the key, fitting into the lock of the trypsin catalytic site. Inhibitors

bind to trypsin, therefore, the trypsin enzyme is not active and cannot produce peptide pheromones. In addition, since the trypsin inhibitor is bound to trypsin, it cannot act at the receptor site for the peptide pheromone. This result provides evidence that the receptor site for pumping pheromone is very similar to the trypsin catalytic site.

The results of this study can give us information on the interaction between the ligand and receptor, and help aid in the identification of analogs used for modulation of receptor activity and to understand how the receptor activates its signal transduction pathway. Peptides triggering behaviors bind to specific receptors in the cell membrane thereby initiating the cascade of events in the transduction of a chemical signal. First, a ligand binds to the receptor. After successfully binding to the receptor, the receptor reads this binding event as a response and translates this to the appropriate sensory system. The specificity of the response depends upon the complexity of the interaction between the receptor and the ligand that results in transduction. The responses from the receptors are then integrated into a behavioral response. In addition, trypsin inhibitors are similar in structure to the peptide pheromones, since they are able to invoke larval release behaviors in their free state.

Saigusa (2000) proposed a model for embryo hatching for the terrestrial crab *Sesarma haematocheir* that involved protease enzymes. Hatching for *S. haematocheir* is highly synchronized with nocturnal high tide (Saigusa 1986a, b, 1992). The hatching sequence for this crab is initiated by a substance released by the parent female called

“hatching-program inducing factor” (HPIF). Ikeda et al. (2006) found that HPIF is released from the female over 3 consecutive nights prior to larval release, with a periodicity close to that of a tidal cycle (~24-25 h). HPIF is also a chemical signal acting as a pheromone, passing through the egg case and attaching to a receptor inside the embryo (Ikeda et al. 2006). The inner layer of the embryo is remarkably digested during the hatching process. Two substances are released from the embryo upon hatching: ovigerous hair stripping substance (OHSS) and a caseinolytic protease (Saigusa 1996; 2000; Ikeda et al. 2006). The hatching substance, OHSS, is found in hatch water. In *S. haematocheir*, ovigerous females treated with hatch water will liberate clusters of premature embryos, not individual embryos (Saigusa 1994). Hatch water for *S. haematocheir* is also affected by the presence of trypsin, since the activity of OHSS was destroyed by addition of trypsin. This result suggests that OHSS is also a protein, and most likely, OHSS is a trypsin-like protease, given that it has a similar molecular weight as trypsin (≈ 20 kDa). The same substance has been found in several other species of crabs, including *S. pictum*, *S. erythroedactylum*, and *Hemigrapsus sanguineus* (Saigusa 1995).

The results of this work support the conceptual model for larval release in decapod crustaceans. The model for larval release in *P. argus* hypothesizes that hatching is controlled by communication between the parent female and the developing embryos. The embryos release peptide pheromones which induce the female to undergo stereotypical behaviors involving the rapid flexing of the abdomen and vigorous

pleopod pumping (Chapter 2, 3). These pheromones originate from the digestion of proteins by trypsin-like enzymes. The receptor for the pheromone is similar to the catalytic site for trypsin because trypsin inhibitor mimics the peptides. Thus, trypsin-like enzymes both produce the peptide pheromones and probably weaken the egg membrane so that it can be easily broken by the females pumping activity that is cued by the pheromones.

6. Endogenous Swimming Rhythms by Phyllosoma Larvae of the Spiny Lobsters *Panulirus argus* and *Panulirus guttatus*

6.1 Introduction

There is an ongoing debate concerning the existence of marine metapopulations, the connectivity of local populations, and the probability of occurrence of self-recruitment (Gaines and Lafferty 1995; Botsford et al. 1998; Sponaugle et al. 2002; Strathmann et al. 2002), which is nourished by the basic difficulty of measuring the flux of small larvae in a complex marine environment. Given the extended larval development time of many decapods, which ranges from weeks to months, it is likely that these species frequently exchange larvae among populations. Conservation and management of marine metapopulations, including spiny lobsters, is likely to be ineffective without knowing how their component parts (spawning sources and recruitment sinks) are connected through larval dispersal.

Larval behavior is a critical component to robust modeling coupling the physics of ocean currents with appropriate biology. For example, the hydrodynamic models that treat coral reef fish larvae as inert particles show no concentration near coral reefs. Only when models include directional swimming at realistic speeds do they reproduce field results (Wolanski et al. 1997). Thus dispersal models that do not include appropriate behavior will fail to predict the dynamics of such highly connected systems.

Palinurid (spiny) lobsters are found throughout the world's oceans between 45°N and 45°S and form the basis of several important commercial fisheries (Phillips et al. 1994; Lipcius and Eggleston 2000). The phyllosoma larvae of spiny lobsters have an extended planktonic duration estimated to last 5 to 12 months (Lewis 1951; Kittaka and Kimura 1989; Kittaka 1994; Goldstein et al. 2006). While developing through a series of larval stages, the pelagic phyllosoma are susceptible to long-distance transport by local and oceanic circulation. It is difficult to study the behavioral processes affecting planktonic survival and transport in the field, since larvae are in low abundance, widely dispersed, and highly cryptic (Phillips et al. 1979; Phillips 1981).

The Caribbean spiny lobster *Panulirus argus* occurs along the east coast of the United States from North Carolina, to the Gulf of Mexico, and throughout the Caribbean to Brazil (Williams 1984). The spotted spiny lobster *Panulirus guttatus* is found throughout south Florida, the Caribbean, Bermuda and Brazil (Robertson and Butler 2003). *P. guttatus* is a small, sedentary tropical lobster that co-occurs with *P. argus*, however, in contrast to the later species which occupies a wide range of habitats, *P. guttatus* is restricted to coral reef habitats and is not found in seagrass or sandy areas far from reef areas. We know almost nothing about the population dynamics or behavior of larval *Panulirus* spp. other than the observation that different larval instars occupy specific depths off the Florida coast (Yeung and McGowan 1991; Yeung and Lee 2002).

Many larvae migrate vertically in the water column on a diel schedule due to an endogenous rhythm and/or specific behavioral responses to environmental cues (Forward 1988; Forward and Tankersley 2001). For example, the first larval stages of many brachyuran crabs share common behavioral traits that promote movement to the surface and maintenance of a position high in the water column (Sulkin 1973, 1975; Forward and Costlow 1974; Latz and Forward 1977; Sulkin et al. 1980), enhancing their initial dispersal from a hatching site. Early-stage larvae of the western rock (spiny) lobster *Panulirus cygnus* perform relatively shallow diel vertical migrations (DVM), which aid in their dispersal offshore due to the differences in current regimes at different depths. Although hydrodynamic conditions have some influence on larval vertical distribution, the primary depth regulatory mechanisms are probably under behavioral control (Sulkin 1984; Forward 1988).

Phyllosoma larvae have a flat, leaf-like body shape that appears compatible for drifting, and are generally regarded as having little or no directed horizontal swimming ability, but they are capable of vertical movements. Previous studies suggest they undergo nocturnal vertical migration in the upper 50 m (Yeung and McGowan 1991; Booth 1994). There are no studies that document the behavior of phyllosoma larvae underlying vertical migration. Only a small number of field studies document the distribution of phyllosoma larvae in the water column (Yeung and McGowan 1991; Lee et al. 1992, 1994; Yeung and Lee 2002; Manzanilla-Dominguez and Gasca 2004).

Phyllosoma larvae may possess a suite of behaviors, including an endogenous swimming rhythm and behavioral responses to environmental factors, such as light, which can be analyzed and quantified in the laboratory. The use of different vertical distribution patterns over different larval stages allows larvae to be dispersed over long distances during their development, and may influence transport of larvae by coastal and oceanic circulation (Sulkin 1986; Lee and Williams 1999).

Although the long larval duration of the phyllosoma favors long-distance dispersal (Thorson 1961), local oceanographic features such as gyres and countercurrents have been implicated in the entrainment and retention of phyllosoma larvae in South Africa (Lazarus 1967), California (Johnson 1971) and Australia (Phillips et al. 1978) for several different species within the genus *Panulirus*. Phyllosoma larvae could possibly undertake active control over advection through diel vertical migration (DVM), by which they encounter currents flowing in opposite directions at different depths and achieve a horizontal distribution conducive to retention (Johnson 1971; Rimmer and Phillips 1979; Phillips 1981). In the Florida Keys, gyres and countercurrents have been observed in areas where *P. argus* and *P. guttatus* are known to spawn (Gregory et al. 1982; Yeung and McGowan 1991), and which might promote retention and ultimately the recruitment of locally-spawned larvae to maintain the large adult populations.

The aim of this study was to determine if the phyllosoma larvae of two species of palinurid lobsters possess an endogenous rhythm in swimming activity that may

promote transport seaward and that could underlie DVM. I examined the vertical swimming behaviors of phyllosoma larvae under laboratory conditions. These results indicate that an endogenous rhythm in vertical migration is involved in DVM for both of these *Panulirus* species.

6.2 Materials and Methods

6.2.1 Larval Rearing

Ovigerous *Panulirus argus* and *Panulirus guttatus* with late-stage embryos (hatching imminent in less than 24 h) were collected from reef locations near Long Key, FL (N 24° 49.567 W 80° 48.884) and transported immediately to the Keys Marine Laboratory (KML). Each female spiny lobster was maintained in an individual aquarium (76 l) containing water collected from offshore (filtered > 5µm; salinity of 35), and were maintained at ~26°C under a 12:12 light:dark (LD) cycle. The onset of the light phase began at 06:00 h. All females were expected to release larvae within 1 day of collection. Larvae were collected in less than 1 h from the time of hatching and placed in small glass aquaria containing filtered offshore water (26.0 x 16.5 x 15.0 cm) in groups of ~200 larvae. Newly hatched stage I phyllosoma larvae were reared under similar environmental conditions as the adults. Phyllosoma larvae were fed newly hatched *Artemia* nauplii for ~ 20 h.

6.2.2 Rhythm Experiment

All experiments were conducted with stage I phyllosoma larvae that were > 1 d old and that were maintained under a 12:12 LD cycle until they were put into constant darkness. Larvae from six individual *P. argus* and six *P. guttatus* females were tested in these experiments, with an equal number of replicates from each brood. Experiments were conducted in a light-tight room at ~ 27°C. After 20 h of feeding, larvae were transferred (in groups of ~40-50) to a clear rectangular lucite column (24.0 x 6.5 x 4.5 cm) a few hours prior to the time for beginning the dark phase. The column was filled with offshore water (> 5 µm filtered, salinity ~ 35) and was covered at the top to reduce evaporation.

I hypothesized that stage I phyllosoma larvae possess an activity rhythm in which larvae will swim at depth during the day and near the surface at night. Swimming behavior was viewed using far-red light for illumination (maximum transmission 775 nm). Since crustaceans are insensitive to far-red light (*e.g.*, Cronin and Forward 1979), these experiments were considered to be conducted in total darkness. The camera was aligned to view three Lucite columns and behavior was recorded with a time-lapse video system (Panasonic model 13050 VHS time lapse video recorder, and Cohu model 4815-3000 solid state camera). Although larvae were not fed during the experiments, there was little observed mortality until about the fourth day.

6.2.3 Data Analysis

Larvae were either inactive near the bottom of the column or were actively swimming up into the water column. Vertical migration was analyzed as the number of larvae present in the top third of the column (8 cm) in each 0.5 h interval. This depth was chosen because the larvae clearly demonstrated vertical swimming if they ascended to this depth.

Swimming was recorded for at least 5 days. Six replicate columns of larvae for each species were recorded, with the data being analyzed using time series for periodicity by a combination of autocorrelation and maximum entropy spectral analysis (MESA) algorithms (Levine et al. 2002). Endogenous rhythm time series were analyzed using autocorrelation to determine if each time series had significant periodicity. Autocorrelation plots that had peaks exceeding the 95% confidence interval were considered to indicate statistically significant rhythmicity at $P < 0.05$. Period lengths were confirmed with MESA, and only those peaks that corresponded to peaks in the autocorrelation plots were considered to be significant (*i.e.*, exceeding the 95% confidence interval). Period lengths were averaged from the MESA calculations for replicate columns and the time of active vertical swimming related to the ambient LD cycle.

6.3 Results

Phyllosoma larvae for both *P. argus* and *P. guttatus* displayed an endogenous vertical migration rhythm with night activity. The general pattern in swimming activity was slightly different between species. *P. argus* larvae had a peak in abundance in the top third of the column near the time of subjective sunset, a decrease in abundance near the time of midnight, and a less pronounced peak in the early morning hours prior to subjective sunrise, followed by a descent during daytime hours (Fig. 26 A-G). This activity pattern of two peaks of activity near the surface at dawn and dusk is indicative of twilight DVM. *P. argus* larval swimming activity decreased with time under constant dark conditions in all experimental columns.

P. guttatus larvae swam vertically during the time of night in the field (Fig. 27 A-G). Unlike *P. argus*, the activity pattern for *P. guttatus* larvae had an increase in abundance in the top third of the column around the time of subjective sunset, consistent swimming throughout the night, followed by a descent near the time of sunrise and little activity during daytime hours (Fig. 27 A-G). *P. guttatus* larvae displayed a nocturnal DVM pattern, with a slight decrease in swimming activity over the time under constant dark conditions. A possible cause for this decrease was due to lack of feeding and subsequent larval mortality, which was evaluated at the end of each experiment and was highly variable, ranging from 16 % to 45% larval mortality per column.

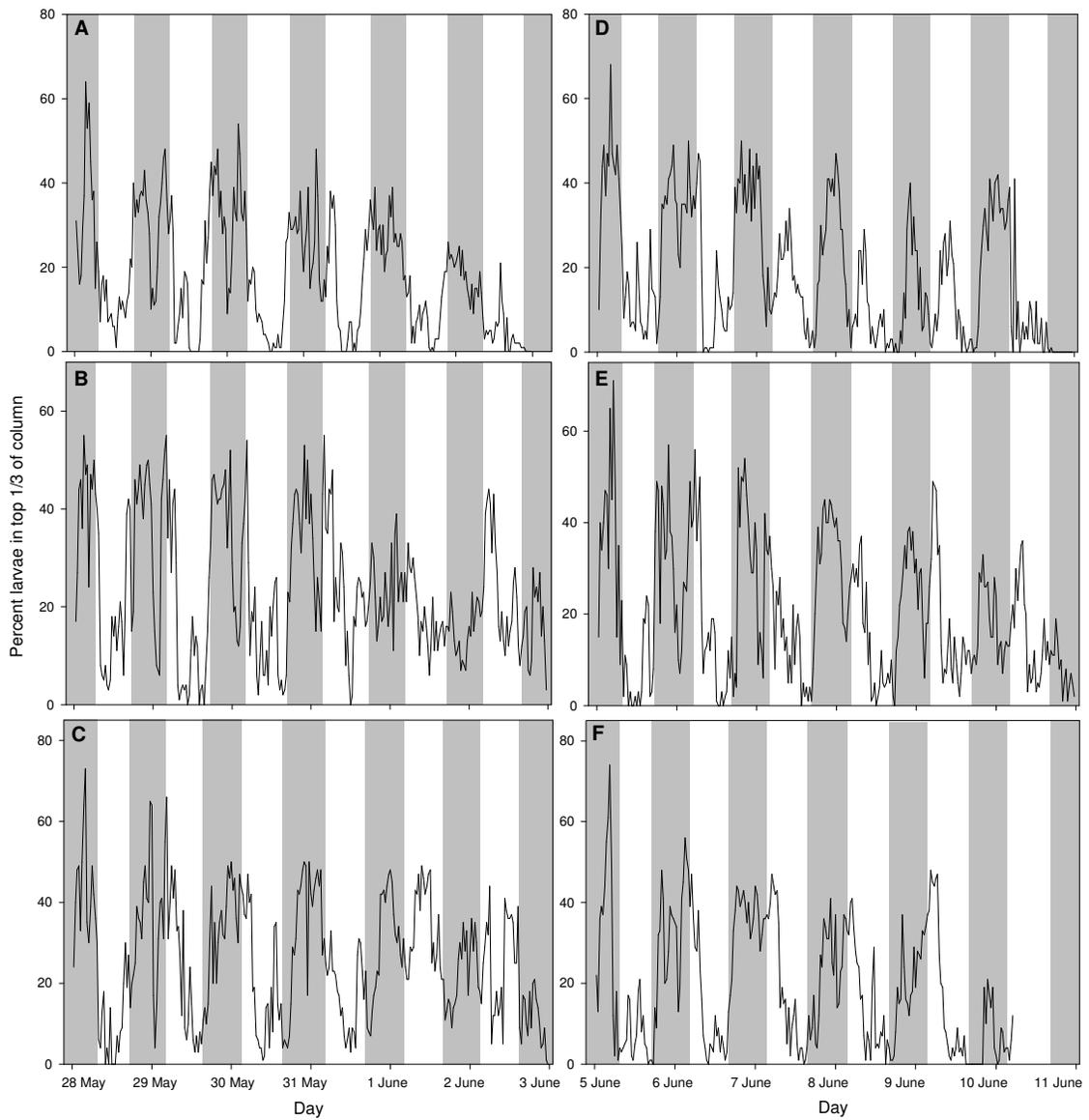


Figure 26: Endogenous vertical migration rhythms of stage I phyllosoma larvae of *Panulirus argus* under constant conditions. Percentage of larvae in the upper third of the column shown as a time series at 0.5 h intervals. Night phase of the LD cycle is represented by gray shading.

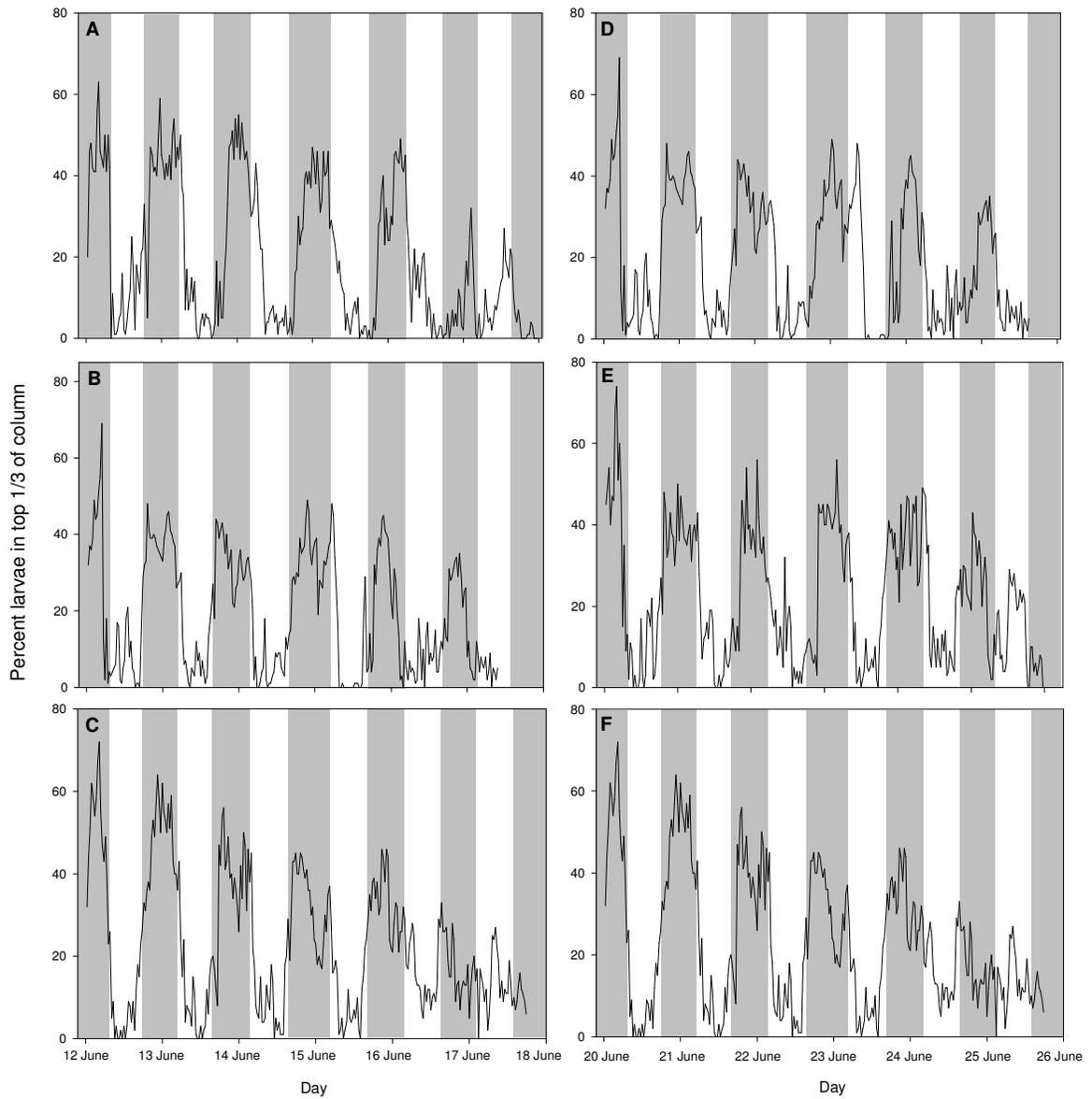


Figure 27: Endogenous vertical migration rhythms of stage I phyllosoma larvae of *Panulirus guttatus* under constant conditions. Percentage of larvae in the upper third of the column shown as a time series at 0.5 h intervals. Night phase of the LD cycle is represented by gray shading.

Significant endogenous rhythmicity in larval swimming was present in all trials for both species. The average free running period for stage I larvae of *P. argus* was 23.5 ± 2.67 h ($X \pm SD$), with a range from 16.1 h to 24.9. The large range in period lengths may be from the observed twilight DVM, in which swimming activity decreased in the middle of the night phase. MESA period estimates for *P. guttatus* ranged from 23.7 to 27.1 h ($X = 24.3$ h, $SD = 1.4$ h). Periodograms for all trials were characterized by single sharp peaks that exceeded the 95% confidence intervals. Activity peaks for both species were not related to tidal times (data not shown). Peak activity consistently occurred during the night phase for both species (Figs. 26-27).

6.4 Discussion

Nocturnal diel vertical migration (DVM) is commonly observed among zooplankton and is characterized by a single daily ascent with minimum depth attained between the times of sunset and sunrise and maximum depth during the day. The biological advantages for an organism that vertically migrates include horizontal dispersion, transport, avoidance of predators, food availability in the upper euphotic zone, breeding, and more efficient assimilation of food at cooler depths (Longhurst 1976; Angel 1985). Light has been found to be the dominant factor in the initiation and control of DVM in the majority of species which display this behavior (Longhurst 1976; Forward 1988). Endogenous activity cycles also contribute to DVM (reviewed by Forward 1988).

The object of this study was to determine whether the phyllosoma of *Panulirus argus* and *P. guttatus* have an endogenous rhythm in vertical swimming that could underlie DVM. For both species of lobster, the swimming activity rhythm persists under constant conditions for at least 6 cycles, indicating that these rhythms are under endogenous control. *P. guttatus* larvae displayed a nocturnal endogenous rhythm in swimming activity, in which larvae were actively swimming at the time of night and were inactive during the daytime hours.

P. argus has a behavioral pattern consistent with twilight DVM, in which larvae actively ascend in the water column near the time of sunset, then descend around the time of midnight and ascend once again in the early morning hours prior to sunrise, followed by a descent during the day phase of the LD cycle. Since larvae were not fed during the experiment, this “midnight sink” response was most likely not due to satiation as commonly observed in copepod species that express twilight DVM (Cohen and Forward 2005). The midnight sink observed for *P. argus* is under endogenous control with descent probably resulting from an activity decrease (passive sinking). The early morning ascent is also under endogenous control, resulting from an activity increase. The descent during the daytime is the result of the inactive phase of the endogenous rhythm. The functional significance of the observed behaviors are hypothesized that *P. argus* larvae ascend to feed at night and descend during the day to avoid visual predation and UV light (Forward 1988).

The results of this study support the hypothesis that an endogenous swimming rhythm underlies DVM and that the LD cycle acts as an entraining agent (Zeitgeber) to synchronize vertical migration with the diel light cycle (Enright and Hamner 1997; Dunlap et al. 2004). Since these rhythms were present in larvae that were reared in the laboratory for less than a day, the endogenous clock that controls this rhythm must have been entrained during embryo development. Pigment in the compound eyes of the embryos is evident 7-8 days prior to hatching (Chapter 2). Thus, there is sufficient time for the embryo to become entrained to the LD cycle. Light is available at the shallow depths where the adults were collected, indicating that the LD cycle is able to entrain the embryos during their development.

P. argus females possess an endogenous rhythm in larval release, in which hatching occurs slightly before the time of nocturnal ebb tide (Chapter 2). The larval release (hatching) rhythm for *P. argus* is controlled by the embryos and is rhythmic relative to the LD and tidal cycle. The embryos possess a circadian rhythm that is clearly entrained to the LD cycle (Chapter 2), and thus the vertical migration rhythm begins immediately upon hatching. As larvae are released, they ascend to the surface during the night phase, and begin to exhibit the twilight swimming pattern of DVM, where larvae actively swim to surface waters, enhancing their dispersal from the hatching site. The same clock involved with controlling the rhythmic larval release behaviors may also

be involved in DVM. Although *P. argus* has a tidal component in larval hatching, it does not continue in the DVM rhythm.

The situation is similar for *P. guttatus*, which display a circadian rhythm in larval release. Embryos are also entrained to the LD cycle, and larval hatching occurs near the time of sunrise (Chapter 2). The endogenous rhythm in vertical migration predicts that larvae will initially move to the surface and be transported away from the adult, until the early morning when they will descend in the water column. Initial upward movement toward the surface is common for many invertebrate larvae and is hypothesized to assist with dispersal away from adult habitats by utilizing the strong horizontal surface currents (Queiroga and Blanton 2005).

Other species of brachyuran crabs possess DVM behaviors related to transport. Larvae of the estuarine crab *Rhithropanopeus harrisi* can become entrained to environmental cycles during embryonic development. Larvae that hatched in the laboratory, but underwent embryonic development in a semi-diurnal tidal estuary, often expressed circatidal rhythms in vertical migrations. If laboratory hatched larvae were reared in an estuary lacking tides, the vertical migrations were more variable. Thus, the vertical migrations of *R. harrisi* larvae appear to be strongly predisposed to entrainment by natural tidal cues (Cronin and Forward 1983, 1986).

As another example, upon hatching, *Uca pugilator* zoeae display a circatidal rhythm in swimming with peaks in activity occurring near the expected time of ebb

currents in the field, with a period length that was consistent with selective tidal transport (STST) that enhance down estuary transport toward local nursery areas. Conversely, *Callinectes sapidus* zoeae did not display a pattern in rhythmic vertical swimming (López-Duarte and Tankersley 2007) upon hatching, but rather, larvae remained near the surface for the 24 h, and then swimming declined under constant conditions. Thus, any swimming rhythm may be under exogenous control and would require regular, periodic input to be maintained.

The vertical distributions and diel migration of phyllosoma larvae are poorly understood, despite the significance of such data for the modelling of transport processes. Published data exist for only a small number of species (*e.g.*, Chittleborough and Thomas 1969; Rimmer and Phillips 1979; Phillips et al. 1979; Branford et al. 2005). *Jasus edwardsii* phyllosoma are primarily found within the upper 100 m of the water column at night, and presumably migrate below this region during the day (Branford et al. 2005). The most pronounced DVM was observed for late-stage phyllosoma, which avoided the 0-20 m surface layer during the day (Branford et al. 2005).

The vertical migration behavior of early stage (I-III) phyllosoma for the western rock lobster *P. cygnus* concentrates larvae at the surface of the water column at night, and at depths of 60 m during the day. Offshore transport vectors generated by wind are maximal at night, and thus early-stage larvae are transported offshore into the southeastern Indian Ocean by the surface wind drift (Rimmer and Phillips 1976; Phillips

et al. 1981; Phillips and McWilliam 1986). The general circulation of the upper 300 m layer flows in the opposite direction toward the western coast of Australia. As the larvae develop, there is an ontogenetic shift in behavior, in which the mid- and late-stage (IV-IX) phyllosoma actively avoid the surface layer because of their increasing sensitivity to light (Rimmer and Phillips 1979). This behavior allows the larvae to be subject to the subsurface circulation which returns them near to the coast of Western Australia (Phillips et al. 1981). Since *P. argus* and *P. guttatus* stage I phyllosoma larvae exhibit DVM behavior in the laboratory, it is likely that this behavior changes as the larvae progress in age, allowing the larvae to be retained in the long-period eddies near the Dry Tortugas and the lower Florida Keys.

Larval swimming behaviors of *P. argus* and *P. guttatus* (e.g., speed, duration, orientation, and depth over the diel period, as well as over ontogenetic development) are still little known despite their importance to transport. Vertical migration has either been ignored in previous attempts to model larval transport and dispersal of *P. argus* and other *Panulirus* spp. phyllosoma larvae or has been based on the migration patterns reported for *P. cygnus*. This chapter highlights the need to use species specific information on vertical distribution when modeling larval transport pathways.

7. Summary and Conclusions

This dissertation investigated the timing of larval release in two sympatric species of palinurid lobsters; the Caribbean spiny lobster *Panulirus argus* and the spotted spiny lobster *Panulirus guttatus*. The major research findings for each chapter are summarized in the following sections.

7.1 Timing of Larval Release

The timing of larval release in two sympatric species of palinurid lobsters, the Caribbean spiny lobster *Panulirus argus* and the spotted spiny lobster *Panulirus guttatus* was investigated in Chapter 2. I examined the phase relationship between larval release and the natural tidal and diel cycles under laboratory conditions. *P. argus* displayed a nocturnal tidal rhythm in larval release, while *P. guttatus* displayed a solar day rhythm. Under constant conditions for more than 10 d, the same rhythms persisted, implying that the larval release rhythm is under endogenous control. Both species of lobsters were placed under a shifted LD cycle advanced by 12 h relative to the natural photoperiod. Each species advanced their larval release rhythm, indicating that the larval release rhythm can be entrained by the LD cycle. *P. argus* releases larvae near the time of nocturnal high slack water (HSW). Nighttime release reduces visual predation, whereas release near the time of HSW prior to the ebbing tide assists larvae with

transport seaward to offshore nursery grounds. In contrast, larval release for *P. guttatus* occurs near sunrise.

7.2 Control of Egg Hatching

The generalization of the current model for larval release in subtidal crustaceans was tested using *Panulirus argus* as a model species. According to the conceptual model, hatching time is controlled by the embryos, which release a pheromone that stimulates the parent female to undergo behaviors that synchronize larval release. Alternatively, hatching could be controlled by the female. Ovigerous spiny lobsters *P. argus* exhibit stereotypic behaviors during larval release, including rapid abdominal extensions and pleopod pumping activity. Pleopod pumping activity was quantified to determine if a female's pumping activity correlates with the developmental state of the embryos. The role of pheromones released by developing and hatching embryos in controlling pumping behaviors was tested by measuring the pumping response of ovigerous lobsters to (1) hatch water, (2) homogenized embryo water, (3) embryo-conditioned water (unhatched late-stage embryos soaked for 20 h), and (4) water containing homogenized post-hatch egg cases. Bioassays were conducted under constant conditions (dim red light) in the laboratory. Spontaneous pleopod pumping activity increased significantly with increasing embryo development. Upon exposure to hatch water, ovigerous lobsters with late-stage embryos displayed increased pleopod pumping with increased treatment concentration. Water individually conditioned with

homogenized late-stage embryos, intact late-stage embryos, and homogenized post-hatch egg cases all induced larval release behaviors in females with late-stage embryos. Ovigerous females with early-stage embryos did not respond to water conditioned with homogenized early- or late-stage embryos. Thus, active substances are released by embryos at the time of hatching and induce the stereotypical pumping behaviors of the female that synchronizes larval release. The results support the model that larval release in subtidal crustaceans is controlled by pheromones released from hatching embryos.

7.3 Role of Peptides

Larval release in *P. argus* is highly synchronous and is controlled by a “pumping pheromone” released from the hatching eggs. The pheromone induces a parent female to undergo stereotypical larval release behaviors, including rapid abdominal extensions and pleopod pumping. These behaviors help to break open the egg membranes and result in the synchronous release of larvae. I hypothesized that larval release behaviors are induced by pheromones comprised of small peptides. I quantified pleopod pumping activity upon exposure to a range of synthetic peptides to identify compounds that will induce larval release behaviors. Chemically-cued pumping behavior was described in terms of the threshold concentration for response, maximum percentage response and effective concentration range. Pleopod pumping behavior was evoked by di- and tripeptides with a neutral amino acid at the amino terminus and a basic amino acid at the carboxy terminus, and also by the basic-basic dipeptide Lys-Arg. All

carboxy-terminal arginine peptides tested produced a significant pumping response, with the exception of Trp-Ile-Arg. Response concentration thresholds ranged from 10^{-9} M for the most potent peptide (Gly-Arg) to 10^{-4} M for the least potent (Gly-His-Lys). The maximum percentage of lobsters responding was largely independent of the threshold concentration and ranged from 24.3 to 58.3 %. Effective concentration ranges for the peptides were variable from 1 to 4 orders of magnitude. Pumping response usually declined with increasing concentration beyond the concentration that evoked the maximum response of the peptides. These results also support the conceptual model that larval release in subtidal crustaceans is controlled by small peptides which act as pheromones.

7.4 Role of Trypsin-Like Serine Proteases

The general model for larval release in *P. argus* proposes that hatching time is synchronized by a pumping pheromone that stimulates an ovigerous female to undergo larval release behaviors. These stereotypical behaviors include rapid abdominal extensions and vigorous pleopod pumping activity. Carboxyl-terminal arginine peptides serve as pheromone mimics that are capable of inducing these behaviors (Chapter 3). I investigated whether these peptides originate from the action of trypsin-like enzymes as part of the egg hatching process. A bioassay was used to measure pleopod pumping activity of ovigerous lobsters with late-stage embryos subjected to increasing concentrations of (1) trypsin, (2) trypsin inhibitor, and (3) a 1:1 combination of

the two. Incubating females in exogenous trypsin caused eggs to be released from the female, and inactive larvae to be released from eggs. Spontaneous pleopod pumping activity increased significantly with increasing concentrations of trypsin. An increase in pumping behavior was observed upon exposure to turkey egg white trypsin inhibitor. Pleopod pumping behavior was terminated when ovigerous females were subjected to a combination of trypsin and trypsin inhibitors. Ovigerous females subjected to hatch water treated with trypsin ceased larval release behaviors. This result suggests that the active site for the pheromone receptor is similar to the trypsin catalytic site. These results support the premise that peptide pheromones are generated by trypsin-like enzymes acting on the egg membranes and that these enzymes assist in the degradation of the egg membranes at the time of hatching.

7.5 Swimming Behavior of Phyllosoma Larvae

Finally, I investigated whether the phyllosoma larvae of two species of palinurid lobsters, the Caribbean spiny lobster *Panulirus argus* and the spotted spiny lobster *Panulirus guttatus*, possess an endogenous rhythm in swimming activity that could underlie diel vertical migration (DVM). I examined the vertical swimming behaviors of phyllosoma larvae under laboratory conditions. Stage I phyllosoma larvae for both species were reared for 20 h before being placed inside an environmentally controlled room under constant conditions. Swimming activity of the larvae was observed for a minimum of five days. Both species displayed behavioral patterns related to DVM of

ascending to the top of the column near the time of sunset and remaining near the top during subjective night time hours. Phyllosoma for *P. argus* undergo a midnight sink in which larvae made a descent in the mid-hours of the night, and then again ascend in the early morning hours prior to sunrise. *P. guttatus* larvae ascended to the top of the water column near the time of sunset and remained near the surface during the night phase, descending near the time of subjective sunrise. The swimming rhythm persisted for at least 5-6 cycles, implying that the swimming rhythm is under endogenous control. These results indicate that an endogenous rhythm in activity plays a proximate role in DVM for both species, and that these species undergo DVM in the field.

7.6 Functional Significance

Rhythmicity in hatching with respect to various environmental cycles enhances the chances of larval survival. An organism's fitness, or its ability to survive and place offspring in future generations, is enhanced by appropriate temporal and spatial regulation of larval release behaviors (Neumann 1981; DeCoursey 1983). The selective forces that are responsible for the evolution of larval release behaviors are only speculative. Evolutionary change operates through an ultimate cause and a proximate mechanism. There may be multiple selective forces that are bound together, and thus these forces may be complex and difficult to dissect from one another.

Several hypotheses have been proposed for the ultimate functional advantages of the observed patterns of synchronous larval release. First, larval release at the time of

high tide may be an adaptation to avoid stressful or lethal salinities, since most decapod larvae are generally intolerant of low salinity conditions that occur during low tide (Costlow and Bookhout 1959; Forward et al. 1982). This is an unlikely scenario for *Panulirus* spps., as larvae are released in coastal areas (Herrnkind 1980; Gregory et al. 1982) where the temperature and salinity remain relatively stable. Second, predation is hypothesized to be an ultimate factor regulating the timing of larval release.

Synchronous release of larvae lowers the risk of predation by “swamping” potential predators (DeCoursey 1983), thereby minimizing the likelihood that the progeny of one female will suffer disproportionately from the predation (Paula 1989). Larval release by *P. argus* and *P. guttatus* support this hypothesis. In addition, *P. argus* releases larvae over consecutive nights, suggesting a ‘bet hedging’ strategy to counter the variation in predation from night to night (Phillipi 1993; Stevens 2006). Third, the release of larvae into ebb tide currents at night enhances the chances that larvae are rapidly removed from the area, minimizing the exposure time to localized predators (Salmon et al. 1986; Morgan 1995). Larval release at the time of the outgoing tide by coastal species leads to dispersal of larvae from the release site to offshore nursery grounds (Saigusa 1982; Christy 1986; Salmon et al. 1986). This hypothesis is supported by the results of this study.

The results of this dissertation are consistent with the proposed model for larval release in subtidal decapod crustaceans. The synchronous hatching events that occur for

P. argus are the result of a feedback loop. The model hypothesizes that for *P. argus*, trypsin-like enzymes are secreted by the embryos near the time of larval release, leading to the formation of peptide pheromones and weakening the egg membrane. As a few embryos hatch spontaneously near the time of larval release, 'pumping pheromones' will be released and are detected by the female who responds by undergoing stereotypical larval release behaviors of tail flexing and increased pleopod pumping. These actions physically break open more egg membranes, freeing more pheromone. As more pheromone is released, the female pumps more rapidly. This positive feedback system results in the synchronous release of larvae which ensures that offspring are placed in a suitable environment at a time when they can best survive.

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- 2005 Duke University Graduate School International Travel Award
- 2004 National Science Foundation International Graduate Research Fellowship
- 2003 National Science Foundation K-12 Teaching Fellowship
- 2002 National Science Foundation Young Researcher's Award
- 2001 1st Place Poster Presentation, Symposium at Dauphin Island Sea Laboratory

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