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Effect of Eyestalk-Ablation on Circulating Ecdysteroids in Hemolymph of Snow Crabs, *Chionoecetes opilio*: Physiological Evidence for a Terminal Molt[1]

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SYNOPSIS. Bering Sea snow crabs (*Chionoecetes opilio*) are a commercially important crab harvested in the Bering Sea. Optimal management of this species requires an understanding of the biology of this crab that is currently incomplete. Fisheries managers apply a continuous growth model in their management of snow crab, which assumes that male crabs increase in size throughout their lifespan. Male snow crabs undergo a morphometric molt that leads to a disproportionate increase in chelae size and it is still debated whether this molt is associated with a terminal molt. This study was conducted to determine whether adult male *C. opilio* are anecdysic. Using current knowledge of the hormonal regulation of crustacean growth, snow crab physiology was manipulated to induce an increase in molting hormones (ecdysteroids). Since female snow crabs are known to undergo a terminal molt after attaining reproductive maturity, we compared ecdysteroid levels in eyestalk-ablated terminally molted females, small-clawed males and large-clawed males. Snow crabs were collected from the Bering Sea and maintained in circulating seawater at approximately 6°C. Animals were either eyestalk-ablated or left intact. Ecdysteroid levels in hemolymph were quantified using an enzyme-linked immunosorbant assay (ELISA). Circulating ecdysteroids were significantly higher in small-clawed male crabs when compared to large-clawed males or terminally molted females. Eyestalk-ablation increased circulating ecdysteroids in small-clawed males, but had no significant effect on circulating ecdysteroids in large-clawed males or in terminally molted females.

INTRODUCTION

Snow crab, *Chionoecetes opilio*, are commercially important crabs living at high latitudes that are harvested on the Atlantic as well as the Pacific coasts. In the Eastern Bering Sea alone, over one hundred thousand metric tons of male snow crabs were harvested each year for over a decade until 1994 when the fishery collapsed and was declared over fished (Otto, 1998). Since only adult male crabs having a carapace width greater than 102 mm are commercially harvested (legal size is 95 mm), the understanding of growth physiology is important to the successful management of this species.

Currently, snow crab management employs a growth model that assumes indeterminate or continuous growth of crabs (Zheng *et al.*, 2002). It is unclear and disputed as to whether *C. opilio* are capable of continued growth or undergo a terminal molt after which external growth does not occur. The first thorough study to describe the terminal molt in *C. opilio* used both a field and a laboratory component to investigate growth (Conan and Comeau, 1986). Their results were disputed on the basis that the timing for investigating the occurrence of premolt in adult males (July to December) was not optimum (Donaldson and Johnson, 1988). A better design for the trawl survey would be to sample from December to March during which time snow crabs are known to enter premolt. Most studies pertaining to growth have been conducted using the Atlantic and Japanese stocks, and these support the terminal molt hypothesis (Conan and Comeau, 1986; Moriyasu and Mallet, 1986; Hebert *et al.*, 2002). A more recent publication fueled the controversy over a terminal molt by describing the molting of functionally mature male Tanner crab, *C. bairdi*, which are a congener species (Paul and Paul, 1995).

Male *C. opilio*, like other crabs in the family Majidae, undergo a morphometric molt during which the chelae grow disproportionate to the carapace resulting in a ''large clawed'' male. This molt is not associated with the onset of reproductive maturity since males with smaller chelae are capable of synthesizing sperm (Watson, 1970; Sainte-Marie and Hazel, 1992; Sainte-Marie *et al.*, 1995). Instead it is thought that this molt is associated with functional sexual maturity, a stage at which males will compete with other males and are able to mate with females; therefore this molt is commonly referred to as the morphometric molt to functional maturity and these crabs are termed adults (O'Halloran and O'Dor, 1988). In contrast, small-clawed reproductive males are termed adolescent. Adolescent and immature crabs are known to molt until they reach adulthood. A complete understanding of growth in *C. opilio* is critical for managing this species as the legal harvesting of large adult males is artificially selecting for smaller sublegal adult males under the terminal molt hypothesis, and may result in further population declines in snow crab.

Hormones regulate crustacean molting and regeneration and this regulation is well described in reviews (Skinner, 1985; Chang *et al.*, 1993; Lachaise *et al.*, 1993; Reddy and Ramamurthi, 1999). In brief, ecdys-

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teroids promote molting and are synthesized by endocrine y-organs (Chang and O'Connor, 1977; Keller and Schmid, 1979; Watson and Spaziani, 1985). Y-organs are under the inhibitory regulation of an eyestalk neuropeptide known as molt-inhibiting hormone (MIH) and removal of eyestalks results in an increased secretion of ecdysteroids (Webster, 1986; Chang *et al.*, 1993) and an acceleration of the molt cycle (Passano, 1965; Snyder and Chang, 1986). In crustaceans that undergo a terminal molt, there is evidence that secretion of ecdysteroids diminishes (Hinsch, 1972; Laufer *et al.*, 2002) due perhaps to the degeneration of the y-organ (Carlisle, 1957). Ultrastructural studies of the isopod *Sphaeroma serratum*, showed that y-organs degenerate upon undergoing the terminal pubertal molt (Charmantier and Trilles, 1979). The activity of the y-organ is known to vary during the molt cycle (for a thorough review; see Lachaise *et al.*, 1993), and eyestalk-ablation was shown to increase y-organ cell volume, which correlated with y-organ activity (Simione and Hoffman, 1975).

The goal of this study was to use a physiological approach to determine whether large-clawed *C. opilio* are capable of entering premolt and therefore molting. In this study, eyestalk ablation of small-clawed and large-clawed males, and mature females was used as a means of inducing premolt and concomitant ecdysteroid synthesis. Circulating levels of ecdysteroids were measured and setogenesis and limb bud regeneration were used to assess molt stage. Crabs that undergo a terminal molt should not be able to regenerate appendages, as this process requires molting.

MATERIALS AND METHODS

Animals

Crabs were obtained from the Eastern Bering Sea in the summer of 2001 and 2002. In 2001 morphometrically distinct large–clawed males and adult females were collected using a commercial charter vessel (Billikin; Unalaska) using standard commercial crab pots. In 2002, small clawed males and mature females were collected during the Bering Sea summer trawl survey south of St. Mathew Island (NMFS). All crabs were brought into Dutch Harbor and packed immediately into coolers after which they were transported by air to the seawater facility at the University of Alaska Southeast. All crabs were maintained in large tanks (Living Streams) with 20 animals per tank in flow-through seawater chilled to 6°C and fed either herring or squid once each week *ad lib.* All crabs were allowed five days to acclimate to laboratory surroundings prior to experimentation. 30 immature males, 30 adult males, and 30 females were used in the eyestalk-ablation experiment. Crabs were not used in the eyestalk-ablation experiment if they were missing more than one appendage or were missing a single chelae.

Chemicals

Buffer reagents were purchased from Sigma Chemicals. Primary antiserum and horseradish peroxidase-conjugated ecdysone was purchased from Dr. Timothy Kingan (U.C. Riverside). Secondary antiserum (goat anti-rabbit IgG) was purchased from Jackson Immuno Research Laboratories. Enzyme substrate solutions were purchased from Kirkegaard and Perry Laboratories, Inc (KPL).

Morphometrics

Measurements were made on all male crabs (n = 73) obtained live from the Eastern Bering Sea. Crabs were tagged at the base of the fourth-walking leg using numbered cable ties. Carapace width (CW) and chelae height (CH) of the right chelae of male crabs was measured excluding the spines to the nearest 0.1 mm. The CW of all females (n = 30) was measured excluding the spines to the nearest 0.1 mm. Shell condition and limb damage was noted.

Hemolymph sampling and eyestalk ablation

Hemolymph sampling began five days after the arrival of the animals into the Juneau seawater facility. Eyestalks were bilaterally ablated from half of the immature and adult males, and females two weeks after hemolymph sampling was initiated using fine iris scissors. Hemolymph (200 μl) was withdrawn from the base of a walking leg and frozen prior to assay. Hemolymph was sampled each week for two weeks and then every day for two weeks immediately following eyestalk-ablation. After the intensive sampling period, hemolymph samples were removed weekly for up to sixty days. Mortality was monitored throughout the length of the experiment.

Hemolymph extraction

Hemolymph (50 μl) was extracted in chilled methanol (75%), centrifuged (10,000 rpm × 10 min), and the supernatant evaporated to dryness (Thermo-Savant). Samples were reconstituted in 125 μl assay buffer (AB/BSA; 25 mm $NaPO_4$, 150 mm NaCl, 1 mm EDTA, 0.1% BSA, pH 7.5) and assayed for ecdysteroids.

Ecdysteroid ELISA

Samples were assayed using an ecdysteroid enzyme-linked immunosorbant assay (ELISA) previously developed using 20-hydroxyecdysone as the standard (Kingan, 1989) and later modified for insect cells (Kingan, 2000). In brief, standards and samples were assayed in duplicate on 96-well plates (Costar) previously coated with rabbit IgG (Jackson Immuno Research Laboratories), blocked with AB/BSA containing 0.002% sodium azide and washed using an automated plate washer (Biorad Model 1575). All assay volumes were exactly 50 μl. HRP-conjugated ecdysone (50 μl; 1:4,000) was added to each well and plates were agitated for 5 minutes at room temperature. Primary antiserum (50 μl; 1:1,000,000) was added to each well except for wells designated for non-specific binding. Plates were agitated for 5 minutes at room temperature prior to overnight incubation at 4°C.

Plates were washed in PBS (3 × 5 minutes). Color development was initiated by adding TMB substrate solution at room temperature (100 μl; KPL). After 15 minutes of agitation at room temperature, phosphoric acid (100 μl 0.1M) was added to stop color development and absorbance of wells was read at 450 nm using an ELISA Plate Reader (Biorad Model 680). The detection limit of the ELISA is 5 pg. Data was analyzed using a one-way ANOVA followed by post-hoc unpaired t-tests (OriginPro 7.5).

Setogenesis

Molt staging was conducting according to a previously described procedure (O'Halloran and O'Dor, 1988). The exopodite of the second maxilloped was removed from crabs that had not yet molted at the end of the hemolymph sampling period to determine the molt stage of individual crabs. Appendages were examined for apolysis and setal growth using a compound microscope (Olympus) and staged as previously described (Aiken, 1973; O'Halloran and O'Dor, 1988).

Limb bud regeneration

The presence of limb buds was determined by close examination of the blastema for the evagination of a protruding bud. In late premolt, the regenerate can clearly be recognized as an opaque protuberance.

RESULTS

Eyestalk-ablation resulted in low mortality among all crabs (only one crab died within 7 days of this procedure). Long-term captivity of crabs held in the laboratory lead to an increased mortality among all groups after 70 days in captivity, which was independent of eyestalk-ablation. There was no mortality during the hemolymph sampling period (50 days) although mortality did increase after 70 days. Most of this mortality was due to unsuccessful and incomplete molting.

The morphometrics of all male crabs is shown in Figure 1 and two distinct groups are demonstrated. One of these groups is represented by males having a large CH to CW ratio (greater than 0.19) and is composed of large-clawed adult males. The range of CW of large-clawed adult males in this study is 50–120 mm. The other group of males has a lower CH to CW ratio (less than 0.18) and is composed of small-clawed males ranging in CW from 40–60 mm. Females were not measured for morphometrics because their claw morphology does not change upon their terminal molt.

Circulating levels of ecdysteroids at the start of the experiment (*i.e.,* before eyestalk ablation) are quite low in females (2.1 ± 0.4 ng/ml), and adult males (3.98 ± 0.8 ng/ml) when compared to immature males (107 ± 22 ng/ml). Concentrations of circulating ecdysteroids did not change significantly throughout the fifty days of the experiment in all of the intact crabs. Figure 2 shows the circulating levels of ecdysteroids over the course of the experiment in intact and eyestalk-ablated small-clawed males (2A), large-clawed males (2B), and females (2C). The dashed vertical line at day 15 represents the time at which eyestalks were ablated from crabs. There is a higher variation associated with circulating ecdysteroids in large-clawed males that we did not observe in the females or the small-clawed males. Eyestalk-ablation had no effect on circulating ecdysteroid levels in either the females or adult males. In the small-clawed males, circulating ecdysteroids increased significantly 5 days post eyestalk-ablation ($P < 0.05$) and this increase becomes more significant by the end of the 50 days ($P < 0.001$).

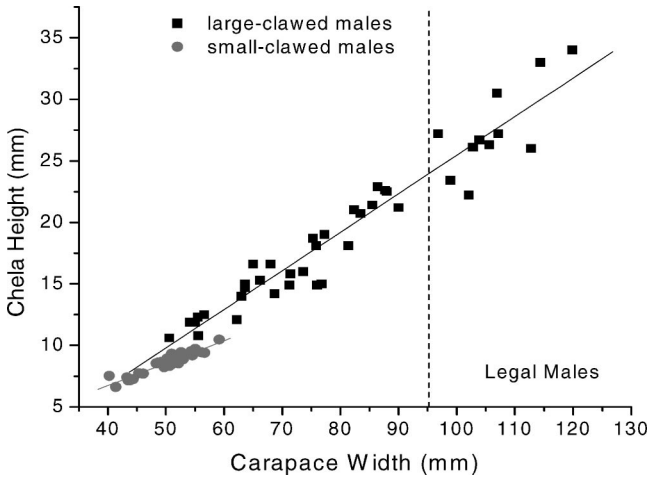

FIG. 1. The relationship between chelae height and carapace width of small-clawed (n = 32) and large clawed (n = 41) snow crab, *Chionoecetes opilio.* Regression line has been drawn through each subgroup of male data to distinguish groups (r^2 for large clawed males is 0.92; r^2 for small clawed males is 0.89; $P < 0.0001$). The dashed line delineates the size at which crabs may be harvested.

Upon completion of the experiment, the molt stage of each crab was determined using setogenesis as described previously (Aiken, 1973; O'Halloran and O'Dor, 1988). Figure 3 shows some of the major characteristics associated with visualizing molt stages, including the retraction of the epidermis during apolysis during early premolt. All of the females and large-clawed males remained in the intermolt stage (C_4) of the molt cycle, whereas each small-clawed male had either attempted to molt or was determined to be at some stage of premolt. Regeneration of any lost appendages only occurred in small-clawed male crabs and could be easily seen by the presence of a limb bud.

DISCUSSION

This is not the first time that hemolymph ecdysteroids have been measured in *C. opilio*, however this study is novel in demonstrating the physiological induction of ecdysteroid secretion through eyestalk-ablation does not occur in terminally molted crabs. Earlier studies showed that hemolymph ecdysteroids are lower in adult males when compared to immature animals (Cormier *et al.,* 1992). Our data support these findings although the levels of hormone that were measured in small-clawed male crabs in this study are ten-

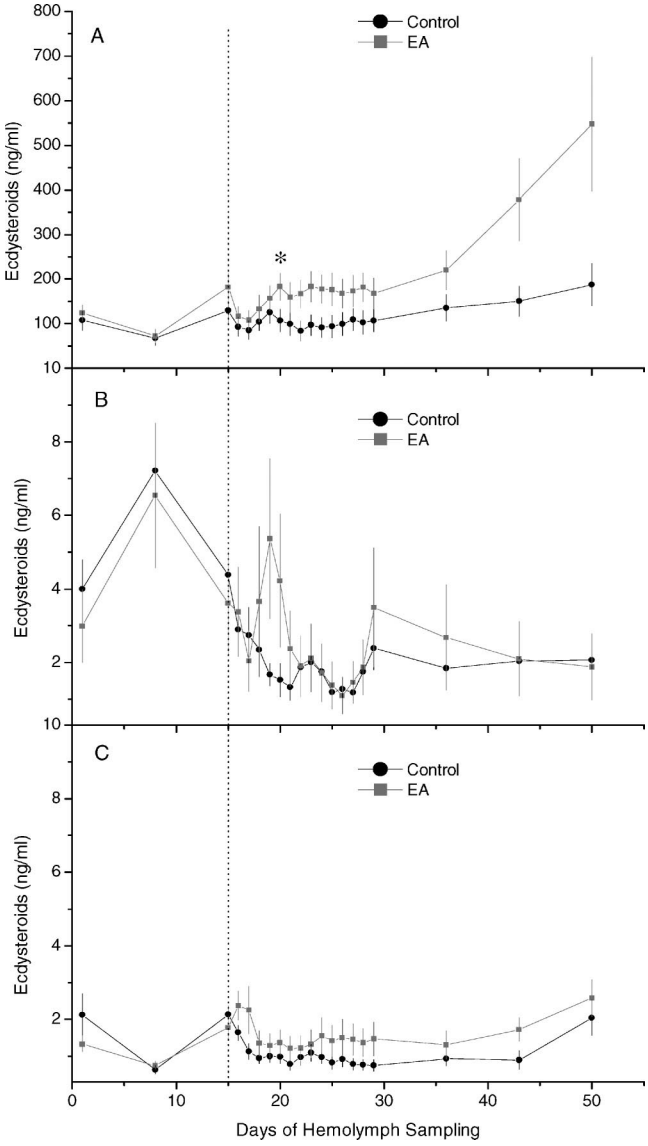

FIG. 2. Circulating levels of ecdysteroids in small-clawed males (A), large-clawed males (B), and females (C). Intact (circles) and EA (squares) are used consistently in A, B, and C. Eyestalk-ablation occurred two weeks after the start of the experiment and is designated by the vertical dashed line through all of the graphs. Hemolymph was sampled every day for 14 days post EA and then each week thereafter. Each data point represents the mean ± SEM of 14 animals. The asterisk in 2A denotes significance at $P < 0.05$.

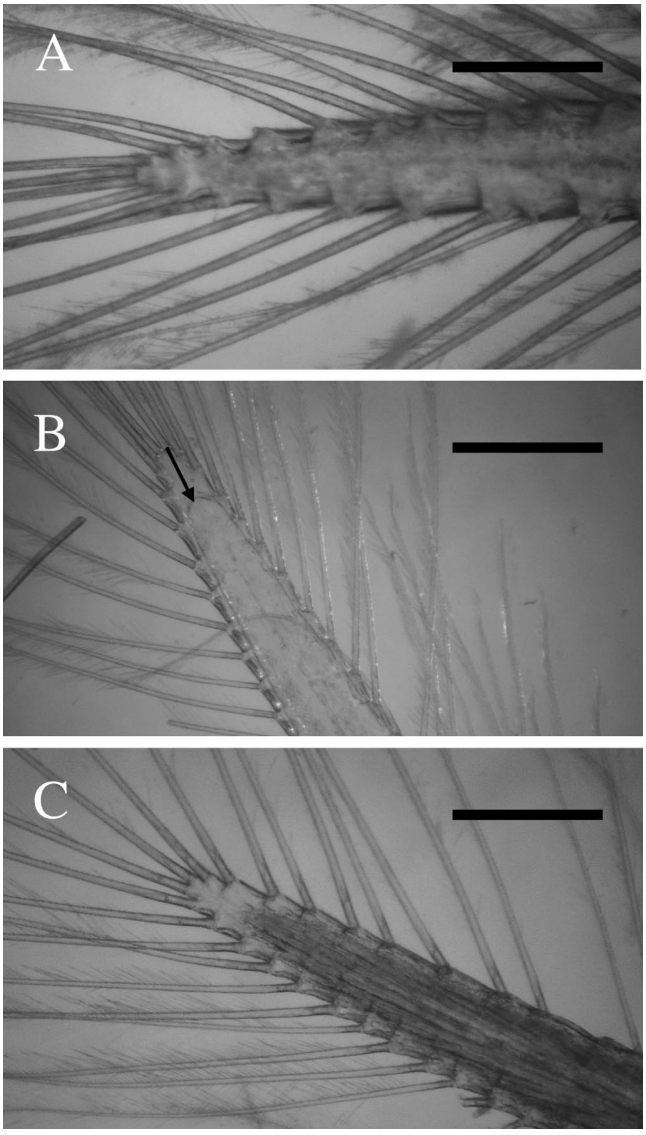

FIG. 3. Molt staging using setogenesis as an indicator of premolt. ''A'' represents an intermolt (C_4) crab. ''B'' represents an early premolt (D_0) stage crab. Note the retraction of the epidermis from the exoskeleton, which indicates apolysis (arrow). ''C'' represents a later stage of premolt (D2). Note the well-developed setae. The exopodite of the second maxilliped was used for molt staging. Scale bar = 0.1 mm.

fold higher than those previously reported. Although collection times varied between these and prior studies (summer *vs.* spring), it is unlikely that this would significantly alter hormone levels.

The levels of ecdysteroids circulating in adult males and females are not significantly different from one another, although it is of interest that large-clawed males exhibit higher variation associated with hormone levels at the start of the experiment. This may be explained due to males of similar morphotypes being at different postmolt stages of their life. Some of these morphologically similar males are newly molted with clean new carapaces, whereas others have not molted for extended periods of time; as visualized by their ''old shells.'' Y-organs from newly molted adult males may have greater activity than those from males that have not molted for years. Ecdysteroids are still measurable in females and large-clawed males, indicating a probable physiological role in reproduction (Reddy and Ramamurthi, 1999).

Terminally molted crabs are incapable of molting most likely due to reduced activity of the y-organ or partial degeneration. Y-organs could easily be removed from small-clawed males whereas it was difficult to locate endocrine tissue in large-clawed males or adult females (S.L.T., unpublished data). The low circulating

levels of ecdysteroids in large-clawed males and adult females support the hypothesis that the y-organ has partially degenerated and lost secretory activity sufficient to induce molting. The data presented in this paper also support that although the y-organ may degenerate, sufficient tissue persists to maintain low levels of ecdysteroid secretion. In response to eyestalk-ablation, only immature males demonstrated an increase in circulating ecdysteroids when compared to intact controls.

Circulating ecdysteroids in immature males were significantly increased five days after eyestalk-ablation (Fig. 2A). The same temporal changes in y-organ activity in response to eyestalk-ablation have been demonstrated in *Cancer magister* (S.L.T., unpublished data). This delay in increased y-organ activity upon removal of the endogenous inhibiting hormone seems surprising since the half-life of MIH-like peptides is less than one hour (Webster, 1996). Increases in y-organ activity may require ''priming events'' such as ultrastructural changes and cellular proliferation, which necessitate time. Changes in y-organ ultrastructure are known to occur during the molt cycle (Lachaise *et al.*, 1993). Stimulation of the y-organs by methyl farnesoate occurred on the order of 48-hours (Tamone and Chang, 1993). In contrast, bioassays for MIH activity clearly demonstrate a rapid inhibition of the y-organs (Soumoff and O'Connor, 1982; Lachaise *et al.*, 1988; Sedlmeier and Fenrich, 1993). The pathways for y-organ stimulation are not as well described as are those for MIH inhibition.

Examination of mouthpart appendages for setogenesis provided significant differences between adults and immature males. None of the adult animals (large-clawed males or females) entered premolt, nor did they show signs of limb regeneration. All of the small-clawed males entered premolt by the termination of the experiment and eyestalk-ablated animals began molting prior to the intact immature males. Statistical analysis of premolt duration did not support the hypothesis that eyestalk-ablation accelerates the molt cycle, however this experiment was not designed to examine this question. Molt stages of immature crabs were not determined prior to the onset of the experiment. It is possible that some crabs in both groups (intact and EA) had already entered early premolt and might respond differently to eyestalk-ablation. Synchronizing the molt cycles of all of the immature animals could have provided more significant results with respect to molt acceleration, but does not change the fact that eyestalk-ablation increases circulating ecdysteroids in immature males.

Immature males range in CW and can reach sizes greater that 120 mm (Otto, 1998). It is probable that in the study that showed functionally mature Tanner crabs molting in the laboratory, the males were actually adolescents of large CW (>110 mm) since allometry was not determined (Paul and Paul, 1995). The basis for functional maturity was determined by the ability of the male to mate with a female in the laboratory and this may not be the most reliable indicator.

It is clear that *C. opilio* undergo a terminal molt that can be characterized by a change in allometric growth. The morphometrics shown in Figure 1 represent only a small number of experimental crabs, although the two morphotypes are distinguishable. Similar graphs have been generated with much larger numbers of individuals (>20,000) to describe *C. opilio* populations in both the Eastern Bering Sea (Otto, 1998) as well as the Atlantic (Hebert *et al.*, 2002). In this experiment, males were presumed to be adults based upon chelae size prior to experimentation. The range in CW of crabs included sub-legal males (<95 mm) to emphasize that a component of the male population will not recruit into the fishery. In fact, the removal of larger males in the fishery may be selecting for smaller males in the population as has been noted for Atlantic populations of blue crab, *Callinectes sapidus* (Abbe, 2002; Kendall *et al.*, 2002). In crabs that demonstrate indeterminate growth, a reduction of fishing pressure on large crabs can lead to a fairly rapid return of large males into a population. But, it has also been demonstrated in fishes, that the removal of large males can influence heritable characteristics (*i.e.*, size) within a population (Conover and Munch, 2002; Resnick *et al.*, 1990). In crabs that undergo a terminal molt, removal of the larger crabs may have consequences on the size structure of the population that are remedied only after reducing the fishing pressure on large males over generational time span.

In summary, male snow crab undergo a terminal molt after which they do not respond to eyestalk-ablation with increased circulating ecdysteroids as do the small-clawed males. This should be of concern to the fishery as the size at which males undergo this terminal molt can vary substantially. It remains unclear as to what factors (genetic or environmental) dictate the size at which a male undergoes this terminal molt.

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