HORMONAL AND ENVIRONMENTAL REGULATION OF THE MOLTING CYCLE IN THE CRAYFISH *FAXONELLA CLYPEATA*[1]

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I. Introduction

The molting process, one of the more interesting aspects of crustacean physiology, has been divided by a number of authors (Drach, 1939; Carlisle and Dohrn, 1953; Travis, 1955a; and Passano, 1960b) into four periods: (1) premolt, a period of active preparation for molt, which includes a gradual thinning of the cuticle and storage in the gastroliths or hepatopancreas of the inorganic constituents needed for hardening of the new exoskeleton, (2) molt, the splitting and shedding of the old exoskeleton, (3) postmolt, a period of rapid redeposition of chitin and inorganic salts to produce a new cuticle, and (4) intermolt, a period in which the exoskeleton is hard and calcification is maximal. In the premolt stage of astacurans, specifically, gradual resorption of the inorganic material in the exoskeleton and deposition of calcium salts in the form of gastroliths in the antero-lateral walls of the cardiac stomach occur.

Darby (1938) has stated that operative injury appears to hasten the next molt in the shrimp *Crangon armillatus*. R. Smith (1940) did not find a shortened intermolt period in the crayfish *Procambarus clarki* when an injury, other than eyestalk removal, was inflicted early in intermolt. On the other hand if the subtropical land crab *Gecarcinus lateralis* lacks many limbs, it will molt despite an unfavorable environment (Bliss, 1956). She found that when six to eight limbs were missing, *G. lateralis* molted on completion of limb regeneration; when only one or two limbs were missing, only one of nine crabs molted.

Several investigators have been concerned with the relationship of light and temperature to the molting process. With *G. lateralis,* Bliss (1954) obtained inhibition of premolt regeneration and growth in specimens maintained under constant illumination. She postulated that this effect was mediated through the eyes. Stephens (1955) found that *Orconectes virilis* responded to daily illumination (20 hours) by an increased tendency to molt. Hess (1941) demonstrated that temperature is a factor that influences molting in *Crangon armillatus.* Specimens did not begin molting until the temperature had risen to approximately 29° C and when the temperature fell below this in the afternoon, molting ceased. Light of 75 ft-c had very little if any effect on diurnal molting in this shrimp. The incidence of molting in the lined shore crab *Pachygrapsus crassipes* was also clearly demonstrated to be associated directly with water temperature. Hiatt (1948) found that exuvial frequency was highest during the summer months and relatively low from November to March.

The molting cycle is under hormonal and environmental control. Brown and Cunningham (1939) found that removal of both eyestalks from the crayfish *Orconectes immunis* caused an acceleration of molting. When the contents of eyestalks were implanted into eyestalkless animals, molting activity was postponed. These investigators concluded that eyestalk tissue liberated a humoral substance into the blood which inhibited molting. Abramowitz and Abramowitz (1939, 1940) working with the fiddler crab *Uca pugilator,* R. Smith (1940) with the crayfish *Procambarus clarki,* Kyer (1942) with the crayfish *Orconectes virilis,* and Scudamore (1942) with the crayfish *Orconectes immunis,* also concluded that the eyestalk was a source of molt-inhibiting hormone. In the crayfishes *Orconectes rusticus* and *Orconectes immunis,* Stephens (1951) found that molt inhibition was obtained with implants of supraesophageal ganglia and circumesophageal connectives. Carlisle (1954) using the green crab, *Carcinus maenas,* suggested that molting was partially inhibited during the molting season by some factor emanating from a source other than the eyestalk.

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Scudamore (1947) suggested that the sinus gland had a retarding effect on gastrolith formation in the crayfish whereas central nervous tissue extracts (supraesophageal ganglia-thoracic ganglia) stimulated gastrolith formation. This observation indicated that central nervous organs outside of the eyestalk were a source of a molt-accelerating substance. In the prawn *Palaemon serratus,* Carlisle (1953) did not find a molt-inhibiting hormone in the eyestalk. Eyestalk ablation led to a significant lengthening of the intermolt period, which suggested that a molt-accelerating hormone was present in the eyestalk. Carlisle and Dohrn (1953) reported that eyestalk extracts of the shrimp *Lysmata seticaudata* contained a molt-accelerating factor.

Gabe (1956) was the first investigator to show that Y-organs, paired, bilateral tissue lying beneath the external adductor muscles of the mandibles, were characteristic features of malacostracans. He suggested that the secretory activity of these glands was correlated with molting. Carlisle (1957) reported that the immediate cause of cessation of molting in the crab *Maia squinado* was degeneration of the Y-organ, which secreted a molt-promoting hormone. Echalier (1959) found that bilateral extirpation of Y-organs caused a definite blockage of growth and of molting in the crab *Carcinus maenas.* Working with the crayfish *Orconectes limosa,* Durand (1960) demonstrated histological changes in the Y-organ. Activity of the Y-organ showed a brief period of activity extending from about three days before molt to four to five days after molt.

Statement of the Problem

The present investigation was undertaken to learn (1) the normal molting cycle of the crayfish *Faxonella clypeata,* (2) whether the molting cycle is influenced by photoperiod, (3) whether temperature is directly associated with incidence of molting, (4) whether loss of limbs shortens the intermolt period, (5) whether a molt-accelerating factor exists in *F. clypeata,* and (6) whether a molt-inhibiting hormone exists in *Faxonella clypeata.*

II. Materials and Methods

The crayfish *Faxonella clypeata* (Hay) is a small crayfish; adults are approximately 15 mm (11.0-19.1 mm) in cephalothorax length. The species inhabits fresh-water ponds, ditches, rivers, and swamps in the Southeastern United States. Ovigerous females retire to burrows. Otherwise members of both sexes are active throughout the year except when the habitat is dry.

The experimental animals were collected with a dip net near Pearl River, Louisiana. The population studied occurred in three shallow pine-land roadside ditches that were exposed to direct sunlight part of each day and contained an abundant plant growth. Two ditches paralleled both sides of Louisiana Highway 41 for approximately 400 yards and a third ditch ran for approximately two miles alongside a logging road that branched off Louisiana Highway 41, thus forming a T-shaped collecting area.

Drought conditions, characterized by complete absence of standing water anywhere in the ditches, and wet conditions, characterized by the presence of standing water throughout the ditches, occurred periodically during the study period. The condition depended on the rainfall (U.S. Weather Bureau climatological data) for the area (Fig. 1). The depth of water in the ditches fluctuated from zero to 34 inches, but an intermediate condition usually existed in which the deeper parts of the ditches were wet and the shallower parts dry. Collections were made only when the ditches contained water. Drought conditions occurred during June, October and November of 1960. Otherwise collections were made at least twice a month.

Black (1958) suggested that a cephalothorax length of 11.5 mm was the lower limit for sexually mature males and females of *F. clypeata.* The data presented herein were obtained from animals with a cephalothorax length of 12.0 mm or longer to eliminate the possibility that animals undergoing pre-maturity molts would influence the determination of molting peaks for the adult population.

Preliminary experimental observations on changes associated with premolt showed that gastrolith formation began within 24 hours after bilateral eyestalk ablation. The appearance of gastroliths indicated that the animals were undergoing premolt and was used in the following experiments as the basis for determining whether premolt had begun. The absence of gastrolith formation was

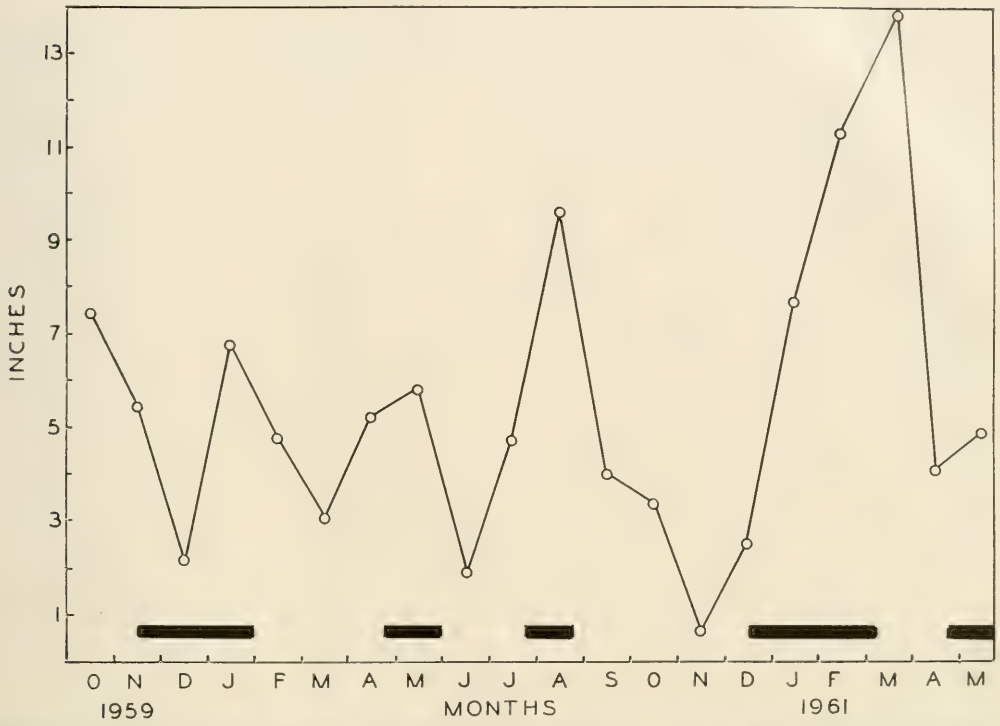

Figure 1. Annual variation in rainfall at Pearl River, Louisiana, expressed as monthly mean number of inches of rain. The solid bars at the base of the figure indicate the period during which 60 per cent or more of the crayfish contained gastroliths.

considered evidence that premolt had been inhibited or had not begun. A soft exoskeleton indicated that the animal had just entered the postmolt phase.

Procedures for statistical analysis of data used in this study were obtained from the book by Snedecor (1956).

III. Experiments and Results

Normal Annual Molt Cycle

To determine the natural molt cycle of *F. clypeata,* collections were made at least twice monthly, except for periods of drought, for 20 months beginning in October, 1959, and ending in May, 1961. A random sample of animals from each collection was dissected to determine the incidence of gastroliths (Table 1). The collections consisted of 9,212 animals and of these, 3,426 were checked for gastroliths.

The collection data (Fig. 2) indicated three major molting periods: fall-winter (November-December-January), spring (April-May), and summer (July-August). Since molting occurred throughout the year, a gastrolith incidence of 60 per cent or above was taken as indication of a molting peak. On only two occasions (September 3 and September 17, 1961) was the percentage of gastroliths below 20. The mean for periods between peaks of molting was 35 per cent with a range of zero to 56 per cent.

Effect of Bilateral Eyestalk Ablation

The precise environmental conditions experienced by the crayfish could not be duplicated in the laboratory. Consequently, measurement of the time between two molts of the same crayfish could not be determined. An experiment was designed to determine the number of days between bilateral eyestalk ablation and the subsequent shedding of the exoskeleton. With the aid of a dissecting microscope, both eyestalks were removed from crayfish by severing the base of the eyestalk with a scalpel. The animals were placed in a dry pan for 10 minutes to allow the blood to coagulate and then they were placed in water. Preliminary experi-

TABLE 1.
Incidence of gastroliths in Faxonella clypeata

Date	With Gastroliths	Without Gastroliths	Percentage With Gastroliths
October 19, 1959	7	18	28
November 7	30	24	56
November 17	23	6	79
November 30	39	39	50
December 22	69	43	62
January 7, 1960	14	2	88
January 21	62	26	70
January 23	36	14	72
February 6	52	63	45
February 17	96	148	39
March 7	32	74	30
March 15	18	29	38
March 19	45	102	31
March 27	17	56	27
April 6	111	100	52
April 24	111	74	60
May 1	101	11	90
May 3	20	8	71
May 13	59	29	67
May 27	15	21	42
July 17	16	9	64
July 23	16	17	48
August 6	78	27	74
August 10	13	3	81
August 16	79	5	94
August 27	48	85	36
September 3	0	5	0
September 17	3	26	10
December 18	13	8	62
December 26	9	4	69
January 1, 1961	28	7	80
January 7	30	2	94
January 21	25	3	89
January 28	18	3	86
February 4	62	11	85
February 18	127	98	56
March 4	104	39	73
March 25	44	144	23
April 16	20	60	25
April 29	50	30	63
May 7	106	77	58

ments showed that animals cooled at 10° C for 15 hours before their eyestalks were removed had a higher percentage of survival than animals that had not been cooled but instead had the stubs electrically cauterized after eyestalk ablation. Forty-six eyestalkless crayfish were kept for 15 days in aquariums containing aerated tap water at room temperatures of 21-24° C. Postmolt crayfish were selected for use in this experiment and were not fed during the course of the observations. The experiment was repeated twice.

In the first five days of the experiment, 83 of 138 animals died before molting and were not included in the results depicted in Fig. 3. By the tenth day all but two molted. They died on the 13th and 15th day, respectively, without having molted. Consequently the percentage of animals molting was 96.4 per cent. The first molt occurred on the fifth day after eyestalk ablation and the last one occurred on the tenth day with a median of 7.9 days for those animals molting.

Influence of Environmental Factors

Light. To determine the effect of daylength on gastrolith production in crayfish, four groups of animals were maintained under different photoperiods from August 9 through October 10, 1960. This experiment was repeated two times, from January 1 to March 18, 1961 and from March 25 to June

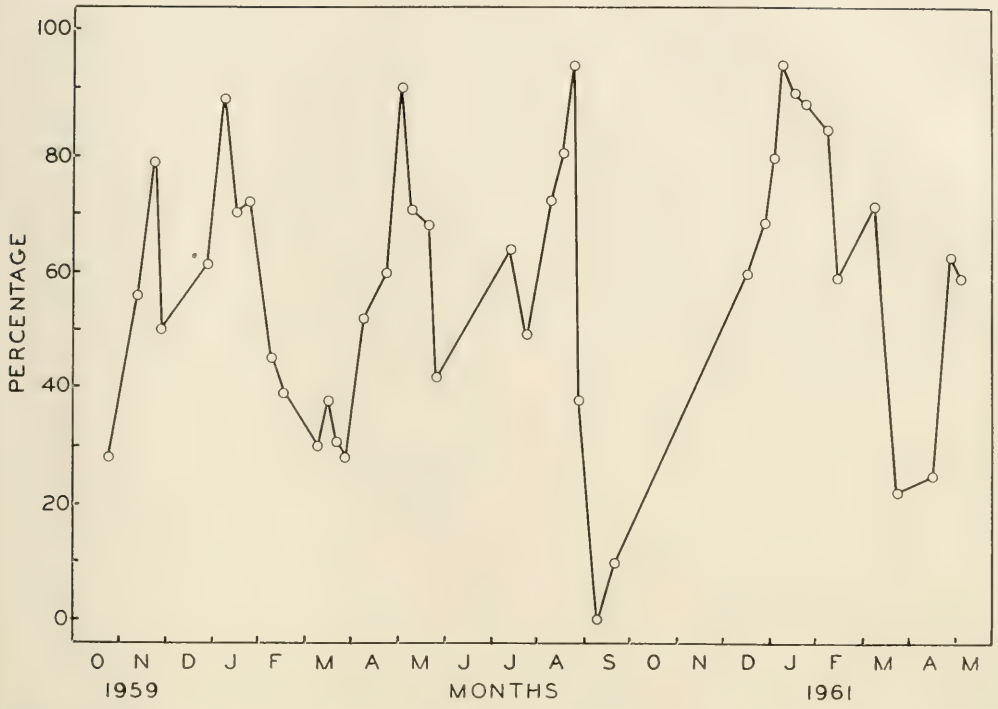

Figure 2. The annual molt cycle of *F. clypeata* expressed as the percentage of animals with gastroliths.

12, 1961. Animals for the first experiment were collected on August 6, 1960; for the second experiment on January 1, 1961; and for the third experiment on March 25, 1961. Males and females were present in each group. The crayfish were maintained in covered rectangular stainless steel tanks, 49 cm long and 37 cm wide, placed side by side in an air-conditioned laboratory. The water was approximately two inches deep and was changed every 15 days. The animals were not fed during the course of the experiment. Illumination was provided in each tank by one frosted 10-watt bulb suspended 20 cm above the water surface. The intensity of illumination at the surface of the water was approximately 40-45 ft-c. Illumination began at 6 A.M. and the duration of light was controlled by automatic time clocks set to provide 6 and 12 hours of illumination daily. Other groups were kept in constant darkness and in constant illumination.

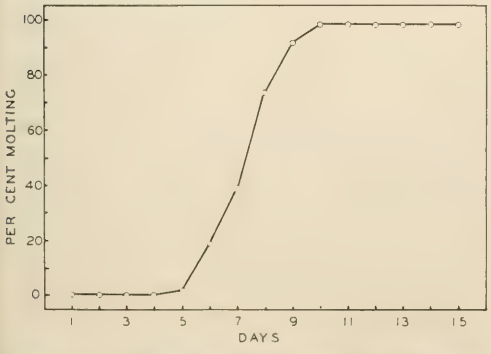

Figure 3. Relationship between the total percentage of crayfish that molted and the number of days after bilateral eyestalk ablation.

All groups started with 90 animals. Group I with mortalities of 33, 61, and 24 per cent in the three experiments respectively was maintained in constant light; Group II with mortalities of 38, 62, and 35 per cent was exposed to 12 hours of illumination; Group III was exposed to six hours of light per day and had mortalities of 44, 68, and 25 per cent; and Group IV with mortalities of 26, 61, and 30 per cent was maintained in constant darkness. A random sample (36, 10, and 24 animals) was selected on the beginning day and a sample (10, 6, and 10 ani-

mals) at intervals of 15 days thereafter for 75 days. The selected animals were removed from each tank and sacrificed to determine the incidence of gastroliths. Exuviae were found when the water was changed but the number of molts per tank was not determined.

The results are summarized in Fig. 4. Group I showed the least tendency toward gastrolith formation. A large peak of gastrolith production was evident in the 15 day sample of the January-March animals and a small peak in the 45 day sample of the March-June animals.

In Group II gastrolith production peaks were noted as follows: 60 day sample in August-October animals, 30 and 75 day samples in January-March animals, and 30 and 60 day samples in March-June animals.

Group III had the greatest tendency toward gastrolith formation. Peaks were as follows: 30 and 75 day samples in August-October animals, 30 and 60 day samples in January-March animals, and a 45 day peak in the March-June animals.

In Group IV gastrolith production peaks were noted as follows: 30 and 60 day samples in the August-October animals, 30 and 60 day samples in the January-March animals, and 30 and 60 day samples in the March-June animals.

The total percentages of gastroliths produced by the animals of each group were: Group I, 41 per cent; Group II, 60 per cent; Group III, 64 per cent; and Group IV, 54 per cent. The differences in behavior between the animals maintained in constant light and Groups II, III, and IV were treated statistically using Student's t test. The means of Group I and Group IV were not significantly different whereas Groups II and III

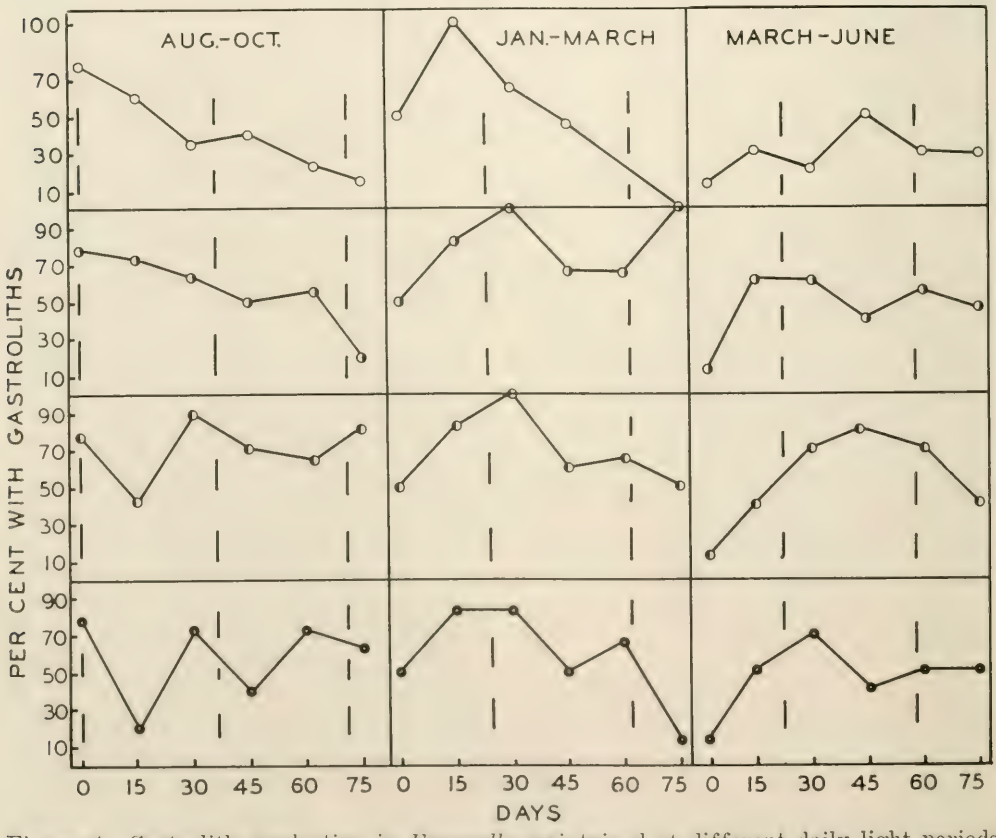

Figure 4. Gastrolith production in *Faxonella* maintained at different daily light periods of 40-45 ft-c.: circles (constant illumination), circles with right half filled (12 hours), circles with left half filled (6 hours), and dots (constant darkness). A probable 40 day intermolt period is indicated by the vertical dashed lines.

were significantly different (p. 0.05 and 0.01 respectively) from Group I.

Temperature. To determine the effects of low temperature on the length of the premolt stage, seven groups of eyestalkless crayfish were placed alternatively between room temperature and a low temperature for varying periods of time. Both eyestalks were removed from postmolt animals as previously described and the eyestalkless crayfish were placed in pans. The pans were white enamel with a bottom diameter 14.5 cm and contained aerated tap water approximately 1.5 inches deep. The room temperature (20-28° C) was maintained by using an air-conditioned laboratory and the low temperature (8-10° C) by placing the animals in a refrigerator. Temperatures were measured by means of maximum-minimum thermometers.

Group I was maintained at room temperature for 24 hours and then placed in the low temperature for 24 hours and then back to room temperature for 24 hours. This alternation of exposure of animals to room temperature and then low temperature was continued until all of the animals had either molted or died. Group II was alternated every 48 hours, Group III every 72 hours, Group IV every 96 hours, Group V every 120 hours, Group VI every 144 hours, and Group VII, serving as the low temperature control, was maintained at 8-10° C for the course of the experiment. Group VIII, serving as the room temperature control, was the batch used above (Fig. 3) to determine the length of time between bilateral eyestalk ablation and shedding of exuviae. The experiment was repeated two times. Animals that died during the course of the experiment were not included in the results.

Table 2 summarizes the results as mean number of days at room temperature before the animals molted. Groups II and IV had the lowest means (7.9 and 7.1 days respectively) and Groups I and VI had the highest means (8.6 and 9.4 days respectively).

The seven experimental groups showed no significant differences; constant low temperatures (8-10° C), however, inhibited the process of premolt to such an extent that the animals did not molt. Analysis of variance by means of the "F" test was used on the results. An F value of 0.90 was obtained which indicated no significant difference among the seven groups.

Influence of Limb Loss on Gastrolith Formation

To determine the effect of limb loss on the incidence of gastroliths, four groups of crayfish with different numbers of limbs missing were kept for a period of 21 days in glass aquariums containing aerated tap water approximately two inches deep. Postmolt crayfish were used and were not fed during the course of the experiment. The aquariums were kept in an air-conditioned laboratory (26-30° C) under identical light conditions.

The chelipeds would undergo autospasy when pressure was exerted on the merus by means of forceps. Walking legs were removed at the base by clipping with fine scissors. The next day 15 animals with the proper number of limbs missing were selected for each group. Group I had one cheliped missing, Group II had two chelipeds missing, Group III had two chelipeds and the first two pairs of walking legs missing, and Group IV having no appendages missing served as the control. The crayfish

TABLE 2.
Number of days at room temperature required for Faxonella to molt after bilateral eyestalk ablation

Groups	No. of Animals	No. of Animals that Molted	Alternate Period in Low Temp.	Mean No. of Days in Room Temp. Required for Molting
I	60	22	24 hours	8.6
II	60	19	48 hours	7.9
III	60	18	72 hours	8.2
IV	60	18	96 hours	7.1
V	60	18	120 hours	8.3
VI	60	18	144 hours	9.4
VII	30	0	continuous	0.0
VIII	138	53	0 hours	7.9

were sacrificed at the end of the experiment (21 days) and the incidence of gastroliths determined. The experiment was repeated three times and the results are presented in Fig. 5 in which Group I represents 49 animals; Group II, 54; Group III, 57; and Group IV, 54.

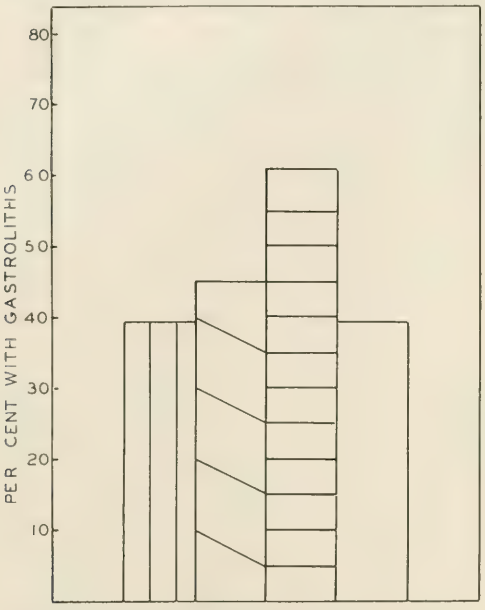

Figure 5. Percentages of crayfish producing gastroliths after appendages were removed. Crayfish with one cheliped removed (vertical line bar), two chelipeds missing (crosshatched bar), two chelipeds and two pairs of walking legs missing (horizontal line bar), and no appendages missing (open bar).

Group I showed a 39 per cent gastrolith incidence, Group II a 45 per cent gastrolith incidence, the control group showed a 39 per cent gastrolith formation, and Group III, having two chelipeds and two pairs of walking legs missing, showed the highest gastrolith incidence, 61 per cent. The number of actual molts that occurred were: Group I, one; Group II, one; Group III, 10; and the control, two.

The above results were treated by a comparison of frequencies using the Chi-square test. Group III, having two chelipeds and two pairs of walking legs missing, showed a statistically significant difference when compared to the control group (p. 0.01). Group II, having two chelipeds missing, did not show a statistically significant difference when compared to the control group (p. 0.30).

In a survey of 753 animals from random collections of groups between molting peaks (Table 3) the following observations were made: 18.98 per cent of the population was either regenerating or would be regenerating limbs, 13.27 per cent had one cheliped missing, 2.39 per cent had two chelipeds missing, 0.93 per cent had two chelipeds and one pair of walking legs missing, 2.12 per cent were regenerating one cheliped, and 0.27 per cent were regenerating two chelipeds.

TABLE 3.
A survey of 753 animals from collections made when the incidence of gastroliths was less than 60 per cent, showing the percentage with missing appendages or those in the process of regenerating limbs

	Number	Percentage
One Cheliped Missing	100	13.27
Two Chelipeds Missing	18	2.39
Two Chelipeds and One Pair of Walking Legs Missing	7	0.93
Regeneration of One Cheliped	16	2.12
Regeneration of Two Chelipeds	2	0.27
Total Number of Animals Missing or Regenerating Limbs	143	18.98

Influence of Endocrine Factors

Molt-accelerating Factor. The following experiments were conducted to determine if a molt-accelerating factor exists either in the eyestalk or in the supraesophageal ganglia and circumesophageal connectives of *F. clypeata.* The experimental animals were selected in a postmolt condition and one eyestalk was removed from each animal 24 hours prior to use in the experiment. Fingerman and Lowe (1957) have found that the chromatophore responses of one-eyed individuals were greater than responses of intact specimens, presumably because the presence of both eyestalks made the crayfish more capable of antagonizing injected chromatophorotropins. The eyestalk is a proven source of molt-inhibiting hormone in the

fiddler crab *Uca pugilator* (Abramowitz and Abramowitz 1939, 1940), in the crayfishes *Procambarus clarkii* (R. Smith, 1940), *Orconectes virilis* (Kyer, 1942), and *Orconectes immunis* (Scudamore, 1942). Preliminary experiments indicated that the eyestalk of *F. clypeata* was a source of a molt-inhibiting hormone and animals with one eyestalk did not molt any sooner than intact animals. The removal of one eyestalk would presumably reduce the titer of molt-inhibiting hormone in the blood and the animal would then be less capable of antagonizing injected molt-accelerating factor.

Eyestalks and supraesophageal ganglia with the circumesophageal connectives attached were removed from only those donor animals having gastroliths. The assumptions were made that those animals having gastroliths were in the premolt condition and if an accelerating factor was present it would be present in greatest titer during this period of the molt cycle.

In the first experiment 40 eyestalks were triturated, suspended in 0.6 ml of van Harreveld's solution (van Harreveld, 1936) buffered to pH 4.8 and 0.02 ml (containing 1.35 eyestalks) of this extract was injected into the third abdominal segment of each of 20 animals. The pH of 4.8 was chosen to determine if an acid solution would activate the molt-accelerating factor. All extracts of eyestalks used in the experiments were centrifuged after trituration to remove the bits of exoskeleton and retinal pigments. Twenty supraesophageal ganglia with the circumesophageal connectives attached were likewise triturated, suspended in 0.5 ml of buffered van Harreveld's solution (pH 4.8) and 0.02 ml (containing 0.8 supraesophageal ganglia with the circumesophageal connectives attached) of this extract was injected into each of 20 animals. The control was composed of 20 animals injected with 0.02 ml each of buffered van Harreveld's solution (pH 4.8).

The animals were placed five to a pan (previously described) under identical light and temperature conditions and were not fed during the course of the experiment. The animals were injected again five and nine days after the original injection. On the 15th day the animals were sacrificed to determine if gastroliths were present. The presence of gastroliths in greater quantity than in the control would indicate that the injections had initiated a premolt condition. The experiment was repeated twice.

The second experiment was conducted in the same manner as described above with the exception that van Harreveld's solution was not buffered and was used at its normal pH of 7.9, this being approximately the pH of the crayfish's blood.

The third experimental procedure was essentially the same as described for the above experiments with the exception that the van Harreveld's solution was buffered to pH 9.6. This pH was chosen to determine if a basic solution would activate the molt-accelerating factor.

The results are presented in Fig. 6. At a pH of 4.8 the injection of the extract of the supraesophageal ganglia with the circumesophageal connectives attached produced a gastrolith incidence of 63 per cent, the eyestalk extract 45 per cent, and the control 37 per cent. In the experiment using a pH of 7.9, the extract of the supraesophageal ganglia with the circumesophageal connectives attached had a 41 per cent gastrolith incidence, the eyestalk extract 39 per cent, and the control 31 per cent. At a pH of 9.6 the supraesophageal ganglia with the circumesophageal connectives attached extract produced a gastrolith incidence of 4 per cent, the eyestalk extract 2 per cent, and the control 5 per cent.

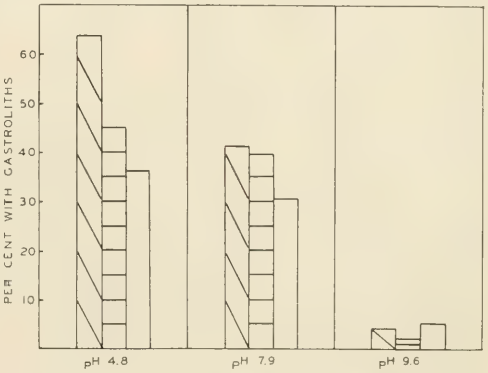

Figure 6. Percentages of crayfish that produced gastroliths after injections of eyestalk extract (horizontal line bar) and supraesophageal ganglia with circumesophageal connectives attached extract (crosshatched bar). The control group (open bar) was injected with buffered van Harreveld's solution.

The above results were treated statistically by comparison of frequencies using the Chi-square test. Injection of the buffered (pH 4.8) extract of the supraesophageal ganglia with the circumesophageal connectives attached showed a statistically significant difference when compared with the control (p. 0.01). There was no statistically significant difference when the results from injection of eyestalk extract (pH 4.8) were compared with the control. The results from injection of extracts of eyestalks and supraesophageal ganglia with the circumesophageal connectives attached did not show a significant difference compared with the controls at pH values of 7.9 and 9.6 (p. 0.30 and p. 0.20 respectively).

Molt-inhibiting Factor. A molt-inhibiting hormone has been found in the eyestalk of several crustaceans (Abramowitz and Abramowitz, 1939, 1940; R. Smith, 1940; Kyer, 1942; and Scudamore, 1942) and some authors (Stephens, 1951; Carlisle, 1954) have also obtained evidence for an inhibiting factor in the supraesophageal ganglia with the circumesophageal connectives attached. The following experiments were conducted to determine if a molt-inhibiting factor exists in the eyestalk or supraesophageal ganglia with the circumesophageal connectives attached of *F. clypeata.*

Eyestalks and supraesophageal ganglia with the circumesophageal connectives attached were removed from only those donor animals not having gastroliths. The assumptions were made that those animals not having gastroliths were either in the postmolt or intermolt condition and if a molt-inhibiting hormone was present it would be present in greatest titer during these periods of the molt cycle.

Eyestalk extracts were prepared as follows: 80 eyestalks were removed from the crayfish, placed in a mortar and triturated. The triturated eyestalks were suspended in 0.6 ml of the appropriate buffered van Harreveld's solution and centrifuged to remove the bits of exoskeleton and retinal pigments. Three buffers were prepared; 0.1 molar sodium phosphate and 0.05 molar citric acid at pH values of 3.5 and 7.6 and 0.1 molar sodium hydroxide and 0.1 molar boric acid to produce a pH of 9.4. These three pH values were selected to determine: (1) if an acid solution would activate the molt-inhibiting hormone, (2) if this hormone could be detected at approximately the pH of the crayfish's blood, and (3) if a basic solution would activate the molt-inhibiting hormone.

Postmolt specimens of *F. clypeata* were selected because these animals did not contain gastroliths. The success of the experiment would be based on the ability of the injected molt-inhibiting hormone to prevent gastrolith production when the eyestalks are removed. The eyestalks were removed as described above. The animals were placed 20 each into two steel tanks (previously described), kept side by side in an air-conditioned laboratory. Water, aerated by means of an air compressor, was changed every other day. The animals were not fed during the course of the experiment.

The extract at pH 3.5 was taken up in a 1 ml syringe and each of 20 animals was injected in the ventral abdominal region with 0.02 ml (containing 2.70 eyestalks). The control animals were injected with buffered van Harreveld's solution (pH 3.5). On the sixth and tenth day the animals were injected again. The experiment was repeated twice. Extracts at pH 7.6 and pH 9.4 were prepared and injected in the same manner as described above.

The procedure for preparing extracts of the supraesophageal ganglia with the circumesophageal connectives attached was the same as for eyestalks with three exceptions: (1) 40 supraesophageal ganglia with circumesophageal connectives attached were used instead of the 80 eyestalks, (2) the extract was not centrifuged, and (3) each 0.02 ml contained 1.6 supraesophageal ganglia and circumesophageal connectives.

The data presented in Fig. 7 are the percentages of animals that contained gastroliths after 12 days other than those animals that died in the first 48 hours after injection. Animals that died after 48 hours and the live animals remaining at the termination of the experiment were dissected to determine the incidence of gastroliths. The presence of gastroliths in smaller quantity than in the controls would indicate that the injections had prevented a premolt condition. In the experiment with eyestalk extracts the results were: pH 3.5, 79 per cent, control 92 per cent; pH 7.6, 57 per cent, control 98 per cent; and pH 9.4, 70 per cent, control 100

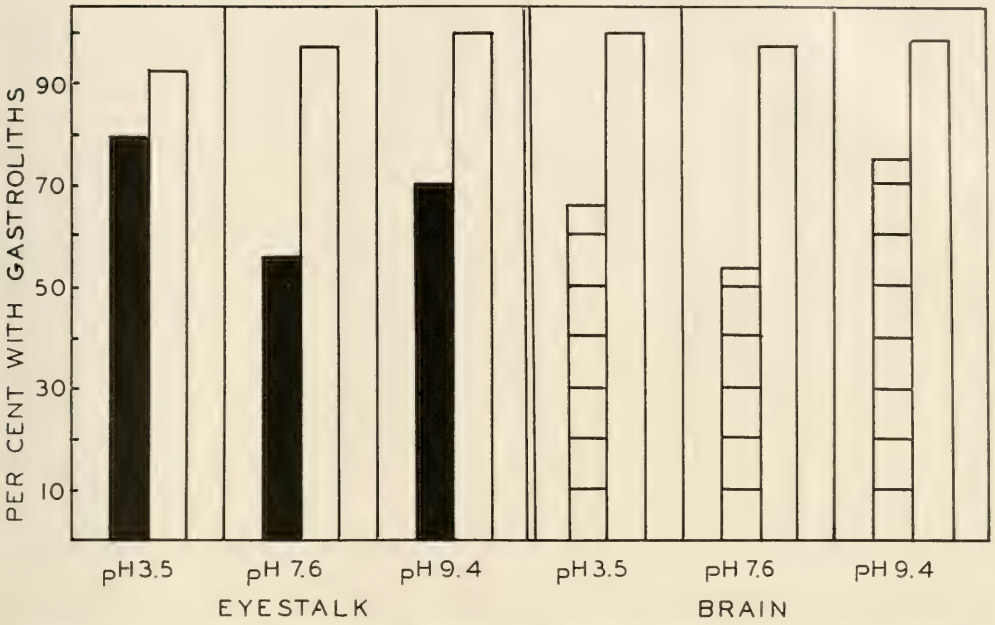

Figure 7. Inhibition of gastrolith formation in *Faxonella* as a response to injected extracts of the eyestalk (black bar) and supraesophageal ganglia with the circumesophageal connectives attached (horizontal line bar). The control group (open bar) was injected with buffered van Harreveld's solution.

per cent. In the experiment with the supraesophageal ganglia and circumesophageal connectives extract the results were: pH 3.5, 69 per cent, control 100 per cent; pH 7.6, 53 per cent, control 97 per cent; and at pH 9.4, 74 per cent, control 98 per cent.

The above results were treated by a comparison of frequencies using the Chi-square test. In the eyestalk and the supraesophageal ganglia with circumesophageal connectives, the inhibiting factor of these extracts at pH values of 3.5, 7.6, and 9.4 showed a statistically significant difference in preventing gastrolith production when compared to the control groups (eyestalk extract, pH 3.5, p. 0.02; remaining extracts p. 0.01).

IV. Discussion

The collection data (Fig. 2), based on per cent of animals with gastroliths, indicated three major molting periods; November-December-January, April-May, and July-August. E. Smith (1953) worked with the growth rate of *F. clypeata* from approximately the same area. She found that maturing females as a group increased significantly in size from February to early June, mid-July to September, and December to January. The male growth pattern was similar to that of the females. Smith's data are substantiated by the gastrolith data in showing that *F. clypeata* undergoes three molts a year.

Rainfall can influence the molting cycle to the extent that animals may be forced to burrow if rainfall is scanty. The animals do not molt in their burrows but wait until they again have ample surface water as was especially noticeable during the winter of 1960-61 when the winter molting peak had shifted approximately 36 days from the period of the previous winter. The amount of rainfall during the months of October and November, 1960, was so small that no surface water collected in ditches, but when the water level again rose after the December rains, the animals began to molt at once after coming out of their burrows (Fig. 1).

R. Smith (1940) found that the intermolt period in the crayfish *Procambarus clarkii* was shorter (average 8.1 days) in eyestalkless animals than in intact ones (28.9 days). Scudamore (1942) determined that the premolt period in the crayfish *Orconectes immunis* was 16.26 days for eyestalkless ani-

mals. Intact animals molted only twice a year (in the spring and summer). The results of R. Smith and Scudamore showed that the intermolt period of eyestalkless animals was shorter than the natural intermolt period. In the crayfish *F. clypeata* the premolt period was 7.9 days after both eyestalks were removed (Fig. 3). The collection data (Fig. 2) indicated that the intermolt period was 60-70 days between the November-December-January molting peak and the April-May molting peak. The intermolt period was 30-40 days between the April-May peak and the July-August peak. Between the July-August peak and the November-December-January peak the intermolt period was 60-70 days. In every case the intermolt period was much longer in intact animals than in eyestalkless ones.

The photoperiod experiment (Fig. 4) showed that exposure of *F. clypeata* to constant light slowed the formation of gastroliths. In reduced photoperiods of 6 and 12 hours the animals had a larger per cent of gastroliths when compared to the animals in constant light. As mentioned above, the normal intermolt period during warm temperatures appeared to be 30-40 days. When a 40 day intermolt period was marked off on Fig. 4, the gastrolith percentage peaks occurred approximately at this interval in groups II, III, and IV. Group I did not follow this pattern but seemed to have a longer intermolt period. This observation was in contrast to the results obtained by Stephens (1955) with a northern group of crayfish. A light period of 20 hours per day during the winter months resulted in an increased molting frequency in *O. virilis*. Stephens postulated that the spring molt in these animals is the result of the transition from darkness or very short daily exposures of light to day-lengths of 12 hours or more when the animals emerge from their burrows in the spring. On the other hand, Bliss (1956) observed that growth in the crab *Gecarcinus lateralis* was inhibited in constant light. Premolt limb regeneration and premolt uptake and retention of water also ceased. Molting did not occur in a constant illumination of 100 ft-c. Suko (1958), working with histological changes of the developing ovaries, found that in the crayfish *Procambarus clarki* the ovaries are influenced by darkness. Secretion of a substance found in the sinus gland and central nervous system that controls ovarian development may have a periodicity. When the quantity of the secretory substance increases to a certain level in the sinus gland, light may be effective in inhibiting the function of the sinus gland.

Constant light may inhibit the production of the molt-acceleration hormone and in reduced light periods of 6 and 12 hours this inhibition would be absent. Once a crayfish begins premolt and then is placed in constant light, the animal will continue premolt but at a slower rate than crayfish at light periods of 6 and 12 hours per day. When the major molting peaks are plotted against day-length (U. S. Weather Bureau data) for the New Orleans area (Fig. 8) molting occurs at the period of short day-lengths and just before and after the longest day-lengths. The April-May molting peak occurs at a day-length of 13 hours and the July-August molting peak at the same length, however at a day-length of 14 hours molting peaks are not found.

In the experiments on temperature, only its role in determining the length of the molting process was considered, not the role of temperature as an activator of molting. The results (Table 2) suggested that once premolt had begun, temperatures of 8-10° C inhibited the actual molting as a result of decreasing the metabolic rate. Mean number of days in room temperature required for molting ranged from 7.1 to 8.6 days for the seven groups. The molting process was inhibited for the time period the crayfish were subjected to low temperatures. No regression in the molting process occurred while the crayfish were chilled. As soon as the animals were placed in room temperatures again, the premolt process progressed until the time the animals were again exposed to an unfavorable temperature.

Passano (1960b) introduced his discussion on temperature effects on crustacean molting with the statement that unlike most environmental variables such as light, temperature can influence both molting itself and molt-control processes. A number of investigators have shown that low temperatures inhibit or lower the incidence of molting and high temperatures increase the incidence (Hess, 1941; Kyer, 1942; Hiatt, 1948; and Passano, 1960a).

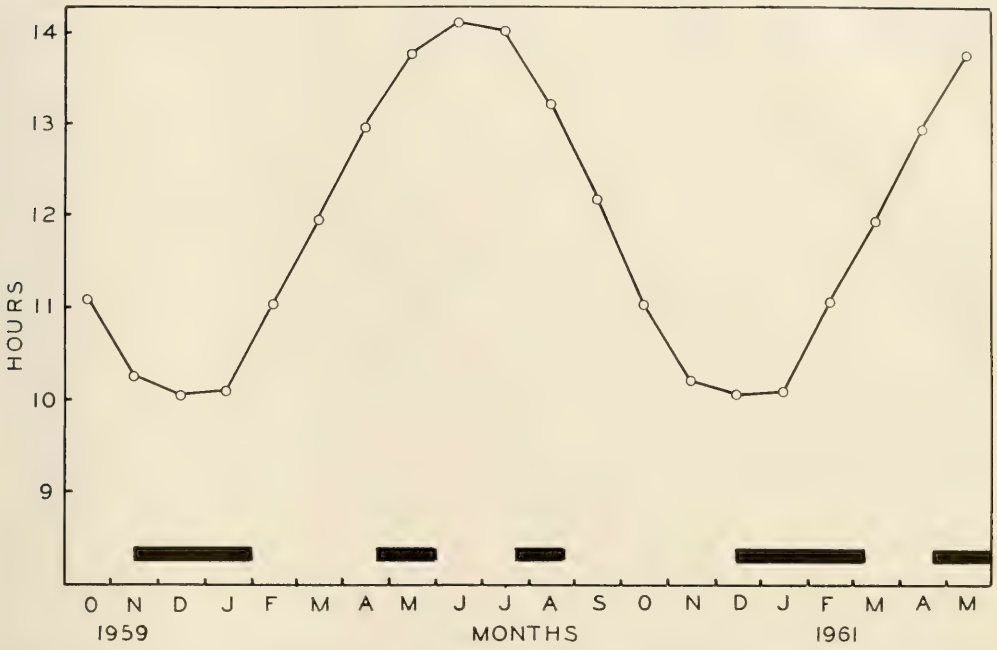

Figure 8. Annual variation in photoperiod at New Orleans, Louisiana, 50 miles southwest of the collection site, expressed as monthly mean number of hours from sunrise to sunset. The solid bars at the base of the figure indicate the period during which 60 per cent or more of the crayfish contained gastroliths.

Diapause in insects is dependent on external factors (photoperiod and temperature) and is followed by molting or hatching. Diapause occurs in any stage of the life cycle except the adult. In the silk worm, *Bombyx mori,* Muroga (1951, cited by Lees, 1955) stated that 40 days chilling is required before it would molt at room temperature. Lees (1955) suggested that from recent experience with the agrotid moth, *Diataraxia oleracea,* and the red spider mite, *Metertetrancyhus ulmi,* temperature should be regarded as a signal stimulus in the same sense as photoperiod. Andrewartha (1943, 1952, cited by Lees, 1955) has shown that if eggs of the grasshopper *Austroiceles cruciata* are chilled at 10° C for 60 days they hatch normally at an incubation of 25° C but if chilled at 6 or 13.5 ° C for the same length of time, fewer eggs hatch when incubated at 25° C. However, normal development occurred when the period was extended beyond the 60 days needed for the 10° C temperature. Williams (1956), working with diapause in the cecropia silk worm, suggested that low temperature served as a catalyst to the brain in restoring the endocrine function.

In the spiny lobster, *Panulirus argus,* Travis (1955a) postulated that molting frequency was a consequence of metabolism. Broekhuysen (1955) found an incubation period of one month was necessary for eggs of the crown crab, *Hymenosoma orbiculare,* at 16.5° C but 38-48 days were required at 12-15° C. A temperature of 10° C blocked premolt in the crab *Sesarma* and when such blockage was removed, premolt proceeded normally (Jyssum and Passano, 1957). Vernberg (1959) showed in the fiddler crabs *Uca pugnax* and *Uca rapax* that temperature affected metabolism. Passano (1960a) hypothesized that a metabolic event was blocked by temperature in the molting of the fiddler crab, *Uca pugnax.*

F. clypeata molted throughout the year and temperatures were never low enough (8-10° C) to block molting completely for any extended period of time. Inasmuch as the U. S. Weather Bureau does not maintain a temperature station at Pearl River, the temperatures recorded (Fig. 9) are from Slidell, Louisiana, eight miles southwest of

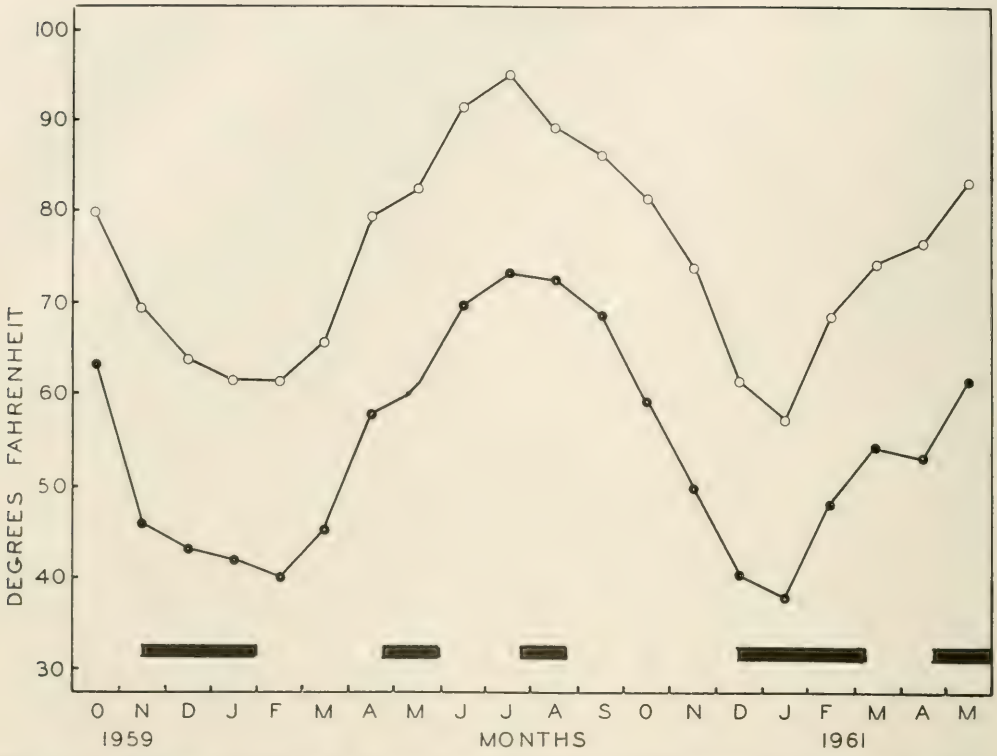

Figure 9. Annual variation in temperature at Slidell, Louisiana, eight miles southwest of collection site, expressed in terms of mean monthly temperatures (circles, high and dots, low). The solid bars at the base of the figure indicate the period during which 60 per cent or more of the crayfish contained gastroliths.

the collection area. Major molting peaks occurred when the temperatures were the lowest for the area and when the temperatures were the highest. The molting periods in both warm and cool temperatures would preclude the conclusion that a chill period such as diapausing insects need is associated with molting in the crayfish *F. clypeata.* The November-December-January molting peak was longer in duration than the peaks occurring in the warmer months presumably because the cold weather (8-10° C) acted as a suppressor. Although a thin film of ice formed over the water surface in January, 1960, and 1961, animals were still present in the water. In insects a cold shock may be necessary before the animal can complete diapause, and thus development, but in the crayfish temperature may act merely to decrease the metabolic rate and not necessarily as an activator of hormonal secretions.

The results (Fig. 5) with *F. clypeata* showed that drastic limb loss was associated with an increase in gastrolith formation. A regeneration stimulus was suggested by Darby (1938) as the reason for the molt-initiating effect of bilateral eyestalk removal. However, later workers have shown that molting was actually initiated by hormones (Brown and Cunningham, 1939; Scudamore, 1942; and Passano, 1953). R. Smith (1940), working with the crayfish *P. clarki,* found that an eyestalk injury was the only type injury that would produce a molt-initiating effect during the early intermolt period. In a sample of five animals, Bliss (1956) produced molting in *G. lateralis* by removing six to eight limbs, but only one out of nine animals molted when she removed one or two limbs. Passano (1960b), however, did not find a molt-inducing stimulus in *Carcinus* on loss of six appendages.

Brown and Cunningham (1939) have shown in the crayfish *Orconectes immunis,* that eyestalk tissue, under nervous control, liberates a hormonal substance into the blood

which inhibits molting. In the crayfish *Cambarus propinquus,* Scudamore (1948) postulated that one reason egg carrying females did not molt was because the presence of the eggs on the pleopods sent an impulse over nerve-reflex pathways that prolonged the molt-inhibiting action of the sinus glands. In *F. clypeata* appendage loss may result in nervous system stimulation which inhibits the sinus gland from releasing the molt-inhibiting hormone, consequently gastroliths may then be formed.

Explanation of activation of the neurosecretory system by loss of appendages may be similar to the thesis used by Fingerman and Fitzpatrick (1956) in which they explained why the fiddler crab *Uca pugilator* exhibited a sexual difference in coloration. In this situation removal of the male's large chela reduced the blood volume to such an extent that the titer of darkening hormone was concentrated and thus produced darkening, however in the female, limb loss did not have as drastic an effect. Appendage loss in *F. clypeata* may have reduced the blood volume to such an extent that the titer of the molt-accelerating hormone was concentrated and thus caused gastrolith formation.

In *F. clypeata* it seems probable that a nervous mechanism is involved in the increased gastrolith formation following drastic loss of limbs. The hormonal mechanism described above requires a serious reduction in blood volume and the loss of two chelipeds and two pairs of walking legs would not reduce the blood volume enough. In *Uca* the large claw was approximately one-third of the body volume. If the production of gastroliths was due to a nervous impulse, then it would seem likely that the loss of two chelipeds did not produce a sufficient nerve stimulus to initiate gastrolith formation, whereas the loss of two chelipeds and two pairs of walking legs did produce a sufficient stimulus.

The loss of appendages may be one of the factors influencing animals to molt between molting peaks. In collection samples from periods between molting peaks, 18.98 per cent of the population contained animals either missing appendages completely or regenerating them (Table 3). Although intact specimens with gastroliths were also found at the same time, limb regeneration may explain why some animals were molting at times other than during the peak periods when the majority of the crayfish molted.

The results from the experiments on the molt-accelerating factors are presented in Fig. 6. In the experiment using van Harreveld's solution buffered to pH 4.8, the results indicated that a gastrolith producing factor existed in the extracts of the supraesophageal ganglia with the circumesophageal connectives attached. Scudamore (1947) suggested the possibility of nervous or secretory factors in or near the central nervous system having a molt-accelerating action. When he injected extracts of central nervous tissue into eyestalkless *O. immunis* and *O. virilis,* stimulation of gastrolith formation and an increase in the rate of oxygen consumption were noted. Carlisle (1953) stated that evidence points to the existence of an eyestalk molt-accelerating hormone in the shrimp *Palaemon serratus.* In 1953, Carlisle and Dohrn published a paper on the shrimp *Lysmata seticaudata* in which they injected intramuscularly an acidified extract of eyestalk tissue and found an accelerated rate of molting.

The results of the injection of extracts of eyestalks and of the supraesophageal ganglia and circumesophageal connectives show that a molt-inhibiting hormone exists in these structures (Fig. 7).

The inhibiting hormone may actually be a series of hormones instead of one hormone. Kyer (1942) suggested that in the crayfish *O. virilis* the inhibiting hormone inhibited an enzyme system involved in the removal from the exoskeleton of calcium and its deposition as gastroliths. Scudamore (1947) has shown that implants of sinus glands into eyestalkless *O. immunis* and *O. virilis* inhibited increase in water content and sinus gland extracts decreased the rate of oxygen consumption after eyestalk ablation. Travis (1951a, 1951b) has shown that during premolt the shrimp *Panulirus argus* took in calcium from its environment, reduced calcium excretion to maintain a normal blood calcium balance, and that the eyestalk played a role in the regulation of phosphate metabolism. Blood proteins of *P. argus* increased prior to molt, declined following molt and reached a subnormal value by the third day. Below normal values remained throughout most of the 14 day postmolt observation

(Travis, 1955b). Durand (1956) stated that there are four cytologically distinct types of neurosecretory cells in the eyestalk and brain of *Orconectes virilis*. The Type 2 neurosecretory cells are the only neurosecretory cells that undergo histologically demonstrable changes in secretory activity in relation to the molting cycle.

Further experiments are necessary before one can make a positive statement that the inhibiting factors originating in the eyestalk and supraesophageal ganglia and the circumesophageal connectives are the same or differ physiologically. Inhibition of gastrolith formation is noted at pH values of 3.5, 7.6, and 9.4, thus showing that the molt-inhibiting hormone can be active over a wide range of pH values. This may indicate that a number of hormones are involved. The possibility also exists that these factors are the same substance but stored in the sinus gland and produced in the supraesophageal ganglia and the circumesophageal connectives. Another explanation could be that there are two different sources for the same hormone, similar to the situation in which the mammalian adrenal cortex produces hormones similar in structure and action to sex hormones, both male and female.

V. Summary

1. The molting cycle of the crayfish *F. clypeata* is defined. While animals can be found with gastroliths throughout the year, three major peaks of molting, November-December-January, April-May, and July-August, occur.

2. *F. clypeata* molts, on the average, 7.9 days after bilateral eyestalk ablation.

3. Exposure of *F. clypeata* to constant light reduces gastrolith production, whereas reduced photoperiods of 6 and 12 hours increase gastrolith production.

4. In eyestalkless crayfish subjected to a temperature of 8-10° C, the molting process is blocked but the process resumes on exposure to temperatures of 20-28° C.

5. Limb loss of at least two chelipeds and two pairs of walking legs causes an increase in gastrolith production.

6. An acidified extract (pH 4.8) of the supraesophageal ganglia with the circumesophageal connectives attached accelerates gastrolith formation when injected intramuscularly into *F. clypeata*.

7. A molt-inhibiting factor occurs in the eyestalks and the supraesophageal ganglia with the circumesophageal connectives attached.

VI. Acknowledgements

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ABSTRACT

The molting cycle of the crayfish *Faxonella clypeata* was defined. Field collections were made at least twice

a month, except during periods of drought, from October 19, 1959, through May 7, 1961. While crayfish contain gastroliths throughout the year in Louisiana, three major molting peaks occur in November - December - January, April - May, and July - August.

The three major molting peaks occur during the warmest and coolest parts of the year. The November-December-January peak is longer in duration than the peaks occurring in warmer months due to the fact that cold (8-10° C) acts as a suppressor. In favorable temperatures the molting process continues until the temperature falls below a value that allows the animal to continue the premolt process.

Constant light reduced gastrolith production, whereas crayfish exposed to photoperiods of 6 and 12 hours per day had a higher incidence of gastroliths than the controls.

Rainfall can influence the molting cycle to the extent that animals may be forced to burrow if rainfall is scant. Animals do not molt in their burrows but wait until they again have ample surface water.

Loss of appendages may be one of the factors stimulating animals to molt during the period between molting peaks. When crayfish in the laboratory lose two chelipeds and two pairs of walking legs, the animals increase gastrolith production in comparison with animals having only one cheliped, two chelipeds, or no appendages missing.

A molt-accelerating factor appears to be present in the supraesophageal ganglia and circumesophageal connectives. An acidified extract (pH 4.8) of the supraesophageal ganglia with the circumesophageal connectives attached is active in accelerating gastrolith formation when injected intramuscularly into *F. clypeata.*

A molt-inhibiting hormone was found in the eyestalks and the supraesophageal ganglia plus the circumesophageal connectives. The experimental results suggest that this inhibiting hormone may in reality be composed of a number of hormones.