A SEROLOGICALLY DETECTED SERUM FACTOR ASSOCIATED WITH MATURITY IN ENGLISH SOLE, *PAROPHRYS VETULUS,* AND PACIFIC HALIBUT, *HIPPOGLOSSUS STENOLEPIS* [1]

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ABSTRACT

An antigenic serum component in maturing female pleuronectids was detected by immunodiffusion techniques. Its presence was related to age, length, and maturity in English sole (*Parophrys vetulus*) and Pacific halibut (*Hippoglossus stenolepis*). The factor had a qualitative seasonal variation for English sole; the highest incidence was during the spawning season and the lowest at midsummer. The factor was detected in all mature female halibut sampled, but complete seasonal data are lacking for this species because samples were not available in summer and fall. The factor was detected in the serum of some immature females of both species during the spawning season. Evidence associating the synthesis of this factor with the production of estrogenic hormones was obtained when estradiol was injected into male English sole and induced them to produce the factor.

Maturity studies are an important aspect of biological investigations of fish. Knowledge of age and size at maturity, fecundity, and duration and frequency of spawning is generally required in the management of a species. As a result of the importance of information on maturity, a wealth of literature exists on the subject covering a broad range of fish species. Most maturity investigations have been concerned with development of ovaries rather than testes because of the importance of egg production in population dynamics.

Biological studies of maturity of the Pleuronectidae (flounders) are representative of the variety of approaches for fish generally. Pleuronectid ovaries may be easily classified as developing or immature by macroscopic inspection during the intervals before and after (and including) the spawning season. Harry (1959) used this method to study time of spawning, length at maturity, and fecundity in three species of flounders. Use of a maturity scale, devised by Heincke (1898) for North Sea herring investigations, allows more quantitative estimates of annual ovarian variations. To calculate the spawning season of the Dover sole (*Microstomus pacificus*) Hagerman (1952) used a typical modification of Heincke's scale, based on grossly discernible criteria such as size, transparency, and presence of macroscopic ova. More precise information can be gained by examining the interior of the ovary. By microscopic study of egg diameter and the development of ova, Thompson (1915) determined the presence of egg stocks for more than one season in the Pacific halibut (*Hippoglossus stenolepis*) and found an extended spawning interval for individual females. Through histological methods, Franz (1910) identified four distinct developmental stages and established a firmer understanding of the maturity process than could be done solely by external observations of the ovary in plaice (*Pleuronectes platessa*).

Characteristic changes in the blood of certain

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female vertebrates are related to maturity and can be studied by biochemical and serological techniques. Serum vitellin has been associated with maturation in hens (Roepke and Hughes, 1935) and related serologically to egg vitellin in fowl (Roepke and Bushnell, 1936). Analogous conditions have been described in other oviparous vertebrate classes including teleosts. Uhlenhuth and Kodama (1914), as quoted by Sasaki (1932), used antisera prepared from carp ovaries to distinguish serum of mature female carp from serum of immature female and male carp. Ridgway associated a serologically detected serum factor of the sockeye salmon (also found at high concentrations in the egg) with maturity in females (Ridgway, Klontz, and Matsumoto, 1962); subsequently he has found the antiserum to cross-react with mature females in all salmonid species tested (Ridgway, unpublished data). Concurrently, Vanstone and Ho (1961), in studies of electrophoretic patterns of coho salmon sera at various stages of development, observed a component that was characteristic of maturing females. Fine and Drilhon (1963) identified a similar protein in *Salmo salar* by immunodiffusion. Existing evidence indicates that serum vitellin may be accounted for by the following sequence of events in oviparous vertebrates: under the control of the pituitary, estrogen produced in the ovary stimulates production by the liver of proteins that are passed through the blood to the ovary and there utilized in yolk formation.

The present study attempts to unite the biological and serological approaches in investigations of the maturity of two pleuronected species. We originally intended to study only Pacific halibut. When we found that collecting an adequate halibut sample was impractical, we included English sole (*Parophrys vetulus*), a more available species. The study is based on a serum-vitellin component, called the HM factor, which occurs in mature female flounders. The objective is to demonstrate that serological methods may be advantageously applied in maturity studies of these species by showing the relation of the factor to various biological features.

METHODS AND MATERIALS

The serological methods, preparation of the antigenic substance used in the methods, and the collection of samples for analysis as well as for determination of age of fish from which samples were taken are described.

SEROLOGICAL METHODS

The procedure for the detection of the HM factor was a microslide adaptation of the Ouchterlony method of double-diffusion precipitin analysis as described by Ridgway et al. (1962). The diffusion method provides a means of identifying antigenic components of a solution through diffusion of the solution and an antiserum towards one another in a semisolid medium.[3] If the antiserum contains antibodies specific for components of the solution, a precipitate line is formed in the zone where given antigen molecules meet specific antibodies in optimal proportions. If two antigen solutions that are placed adjacently diffuse towards a single antiserum source, precipitate lines for common antigen-antibody systems will fuse.

Tests for the presence of the HM factor were made in the manner illustrated in figure 1. Sera from known HM-positive females were placed in positions 1 and 4, thus placing each unknown serum adjacent to an HM-positive individual. Distinct positive reactions are seen in positions 5 and 6; a weak positive reaction in position 2, and no reaction in position 3. This arrangement was particularly useful for positive individuals with low concentrations of HM antigen. Although a distinct line was not necessarily formed, a bending of the control line towards the unknown position, as in position 2, indicated the presence of the HM factor and allowed a highly sensitive test for the HM factor's presence.

Relative concentrations of the HM factor were determined by a single-diffusion method described by Hayward and Augustin (1957). In this method the antiserum is incorporated into the agar at 5 percent concentration and serial dilutions of the fluid bearing the HM factor are introduced into wells in the agar. The end point, the highest dilution at which a visible ring can be observed around the well, is referred to as the titer of the solution for the HM factor. Figure 2 illustrates the reactions of serial dilutions of HM factor from an extract from eggs of starry flounder (*Platichtys stellatus*) with the antiserum used in this study. (The preparation of both the extract and the antiserum is described below.) The end point is at the 1/160 dilution.

[3] The medium in this study had the following composition: Difco agar, 1.5 percent; sodium chloride, 0.72 percent; sodium citrate, 0.6 percent; merthiolate, 0.01 percent; trypan blue, 0.01 percent. The pH was adjusted to 6.7.

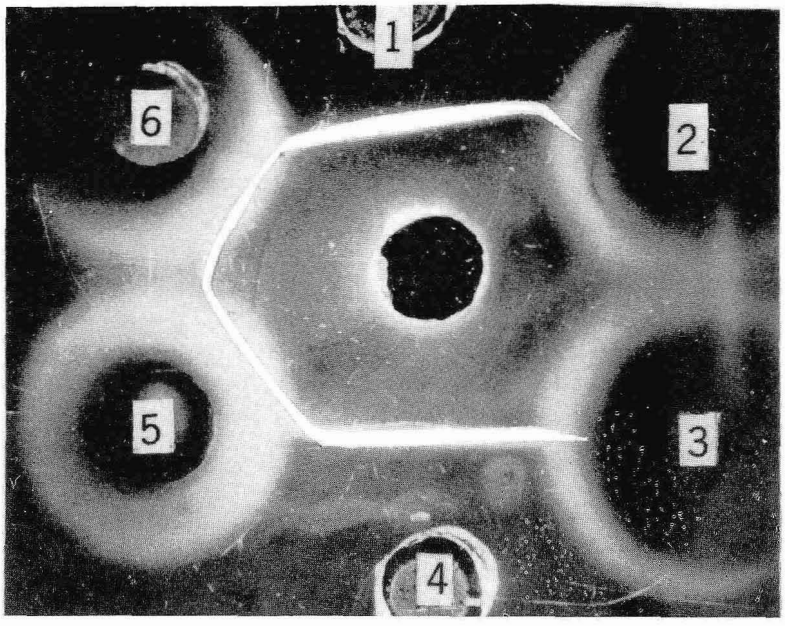

FIGURE 1.—Reactions on typical double-diffusion slide demonstrating tests for presence of HM antigen in kidney-tissue fluids of female English sole. Magnification 5X.

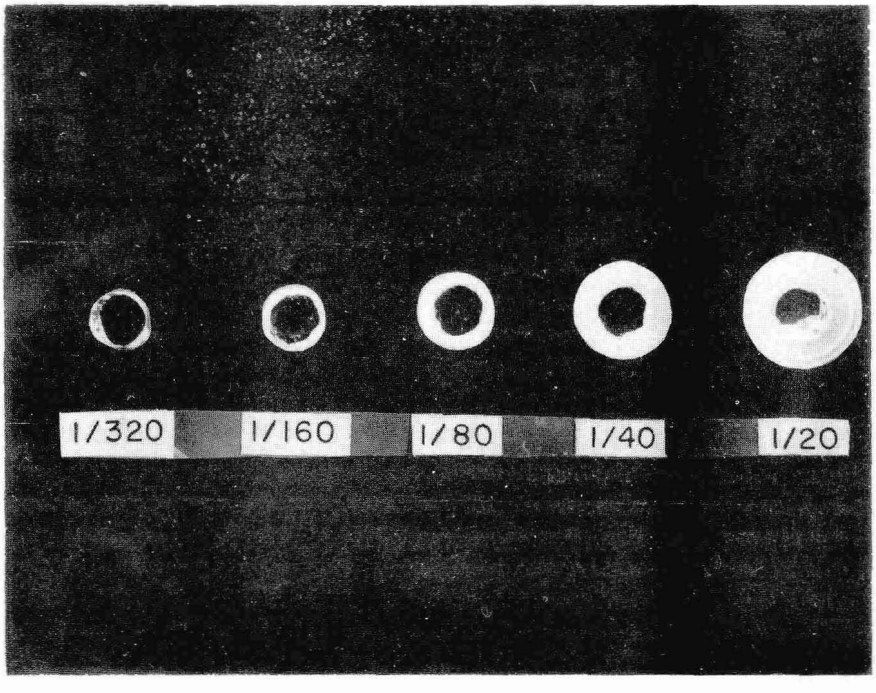

FIGURE 2.—Demonstration of single-diffusion technique to estimate relative concentrations of HM antigen. End point is seen at 1/160 dilution. Magnification 2.5X.

PRODUCTION OF ANTISERUM

The antigenic substance used for production of antisera was a vitellin preparation from starry flounder eggs. This preparation met the classical biochemical criterion for vitellin, since it was the water-insoluble fraction of the egg yolk (Jukes and Kay, 1932). The preparation was made in the following manner: extraction was in a Waring Blendor [4] from 1 part of eggs to 3 parts 1 percent saline solution. One part of the supernatant obtained after centrifugation at 10,000 r.p.m. for 20 minutes was diluted with 11 parts distilled water, and the resulting precipitate was dissolved in 1 percent saline, reprecipitated, and redissolved. The vitellin preparation was utilized in this final form for immunization procedures. Although different injection routes were used and the vitellin preparation was injected both with and without adjuvants, consistently uniform antisera were produced in the five rabbits that were stimulated. Because of this uniformity, a single pooled reagent composed of numerous bleedings from all of the rabbits was made and used throughout this study.

The antibody specificities appeared to be directed toward one or more of the starry flounder's vitellin antigens. The pooled antiserum cross-reacted with serum of mature females of all pleuronectid species tested. Reactions with sera from males were observed only infrequently. These reactions were invariably weak and are discussed in a later section.

COLLECTION OF SAMPLES

Collection dates and numbers of English sole sampled are listed in table 1. All samples were collected by the University of Washington research vessel *Commando* at the Port Orchard area of Puget Sound. Only female English sole were taken. These fish, with the exception of the March sample, were randomly sampled over a variety of lengths. This sample was biased toward smaller individuals with relatively undeveloped ovaries. This bias was based on the relatively high frequency of the HM factor in smaller females in the two previously collected samples; the data for the March sample reflect this bias. Random sampling was resumed through the remainder of the investigation.

Collection of an adequate blood sample from English sole was found impractical, and all qualitative determinations for the HM factor were made from the fluids expressed from kidney tissue. When quantitative data were required, blood samples were collected from larger individuals by cardiac puncture.

The collection data for the halibut samples are presented in table 2. A sufficient blood volume was available from the individual halibut to use serum for determination of the HM factor, although parallel samples of kidney tissue were collected from most fish. All samples were collected under the direction of the International Pacific Halibut Commission.

TABLE 1.—*Collection dates and number of female English sole in samples, Port Orchard area, Wash.*

Collection date	Fish	Collection date	Fish
1962	*Number*	*1963*	*Number*
Dec. 27	30	July 3	25
1963			
Feb. 5	38	July 23	41
Mar. 7	63	October 22	55
June 4	42	November 23	58

TABLE 2.—*Date and areas of collection, number of individual samples, and source of HM antigen for halibut*

Collection date	Area	Fish		Source of HM antigen	
		Female	Male	Serum	Kidney
1960		*Number*	*Number*		
Mar. 25	Queen Charlotte Sound	27	13	x	—
1962					
May 11	Cape Flattery	6	10	x	x
June 6[1]	Queen Charlotte Sound	41	0	x	x
1963					
Feb. 2	Cape Flattery	7	6	x	x
May 23	Cape Flattery	6	15	x	x

[1] Serum collected day of capture; fish eviscerated and iced at this time and kidney fragments collected in port 4 days later.

AGE DETERMINATIONS

Age determinations in both halibut and English sole were made from otoliths. Personnel of the International Pacific Halibut Commission made age determinations of the halibut. English sole otoliths were treated in papain as described by Pruter and Alverson (1962) and read by the senior author.

COMPARISONS OF HM CONCENTRATION IN SERUM AND KIDNEY FLUIDS

The need to use kidney tissue to obtain qualitative data on the HM factor in English sole required us to compare concentrations of the HM factor in kidney-tissue fluids and blood serum of the same individuals.

[4] Trade names referred to in this publication do not imply endorsement of commercial products.

Table 3 makes such a comparison in 10 females taken a few weeks preceding the spawning season. An end-point fluctuation of plus or minus one serial dilution can be anticipated as part of the experimental error inherent in the technique (Kabat and Mayer, 1961). Only one fish exceeded this range.

TABLE 3.—*Comparative titers of HM factor in kidney-tissue fluid and serum of 10 English sole*

Fish number	Kidney fluid titer*	Serum titer*
1	32	64
2	32	64
3	64	64
4	64	64
5	64	128
6	32	32
7	32	32
8	64	32
9	32	128
10	32	32

*Reciprocal of last positive dilution.

In 19 female halibut, where kidney fluid and serum samples were obtained from freshly caught individuals, identical qualitative results were observed for every fish. In the halibut sample taken during June 1962, kidney fragments were obtained from carcasses that had been eviscerated and iced for 4 days, but serum samples were obtained from the same fish when freshly taken. Qualitative tests were made with both serum and kidney fluids. Quantitative tests were made with those sera which gave positive double-diffusion reactions (table 4). The only disagreements between the qualitative data for the kidney-tissue fluids and serum were the three individuals with the lowest serum concentration. The 4-day icing of the cleaned halibut carcass doubtlessly diluted the HM concentration in the adhering kidney fragments; it seems likely all tests would have agreed if the kidney tissue had been fresh.

TABLE 4.—*Comparison of HM titer of blood serum in halibut with double-diffusion reaction of kidney-fragment fluids collected from the same individuals 3 to 4 days after evisceration and icing*

Fish	Serum titer*	Kidney fluid reaction	
		Positive	Negative
Number		*Number*	*Number*
6	256	6	0
7	128	7	0
4	64	4	0
1	32	1	0
2	16	2	0
1	8	0	1
1	4	0	1
1	2	0	1
18	No reaction	0	18

*Reciprocal of last positive dilution.

The above evidence indicates that serum and freshly taken kidney-tissue fluids can be used interchangeably with considerable confidence for detection of the HM factor in these two species when qualitative data are desired.

ANALYSIS OF DATA ON ENGLISH SOLE

The six stages used by Hagerman (1952) to describe development of the ovary in the Dover sole were modified in the following manner to describe the development in the English sole:

Immature:

A. Ovaries very small (generally less than 1 g.), white, transparent, and somewhat gelatinous.

Mature:

B. Developing. Ovaries enlarging, becoming yellowish and opaque. Developing egg visible macroscopically.

C. Gravid. Ovaries very full of yellowish granular eggs.

D. Spawning. Ovaries full of transluscent eggs which run under slight pressure.

E. Spent. Ovaries flaccid; ovarian membrane vascular and sac-like.

F. Resting. Ovaries firm, white, transluscent, and somewhat gelatinous. Distinguished from stage A by the greater size.

The scale was not universally applied in this study owing to the overlap among the various stages. Stages A and F, in particular, were often difficult to distinguish; however, in November through February, including the peak of spawning in December and January (Holland, 1954; Harry, 1959), the stage A ovaries were distinct because stage F ovaries were lacking. All individuals taken during this period with ovaries in stages B through E were HM positive.

Certain individuals with stage A ovaries surprisingly were HM positive during the spawning season (table 5). Maturity classifications were made on these gonads, which were fixed in formalin. When state of maturity is not listed, the ovaries had been sectioned for histological examination before any external maturity classification was attempted. The two individuals with mature gonads had ovaries in

stages B and C, and all gonads classified as immature represented stage A. The HM-positive individuals with stage A gonads from the November samples might have matured later in the spawning season. It is unlikely, however, that the two HM-positive fish with immature gonads from the February sample would have spawned during that season, since all ovaries collected subsequently, through the July sample, were immature or spawned out.

Preliminary results of histological studies being carried out on English sole gonads by Kathleen Ladue, of our staff, were available for the lower two fish from the February sample listed in table 5. Although both were HM positive, only the ovary of the last individual showed atretic follicles as evidence of having spawned; the ovary of the other gave no histological evidence of maturing ova. This evidence supports the previous implications that the HM factor occurs in certain female English sole during the spawning season or seasons preceding that in which they are destined to spawn initially.

TABLE 5.—*HM-positive female English sole from November, December, and February samples that had gonads weighing less than 1 g., Port Orchard, Wash.*

Month of capture and maturity classification	Gonad weight	Standard body length	Age group
1963	*Gm.*	*Mm.*	
November			
Immature	0.2	207	III
Immature	.5	208	III
Immature	.5	225	III
Mature	.7	249	II
Immature	.7	250	III
Immature	.7	257	III
Immature	.9	265	III
1962			
December			
	.8	254	III
Mature	.9	245	II
1963			
February			
Immature	.5	247	II
Immature	.6	235	II
	.8	268	II
	.8	254	II
	.8	265	III

Table 6 lists the frequency of the HM factor in the various samples by age. The samples are grouped by age to follow the ovaries from the developing stage through the resting stage. Samples taken after the height of the spawning season (December and January) through July are assigned to the age they had during the spawning season, because up to July the ovaries are progressing towards the resting stage. The October and November samples are assigned to the age that they would have had at the next spawning season, because the ovaries are maturing during these months. Thus a fish entering its fourth year of life in January would be included in age-group III if taken in July, and age-group IV if taken in October.

The HM factor occurred initially in age-group II in the fish sampled in this study. The possibility of its presence in group-I fish is not ruled out because insufficient numbers of the group were sampled.

At a given age the highest frequency of the HM factor appears in the December sample at the peak of the spawning season. Frequency generally decreases through July but increases again in October and November. The occurrence of a lower HM frequency in II- and III-group fish for the March sample than for either the June or July samples is very likely the result of the bias described under Methods and Materials regarding the collection of that sample.

TABLE 6.—*Frequency of the HM factor in the various age groups of female English sole, Port Orchard, Wash.*

Month and item	Age group				
	I	II	III	IV	V[1]
1962					
December:					
Total number of fish		5	14	8	
Number positive		4	12	8	
Percentage positive		80	86	100	
1963					
February:					
Total number of fish	1	34	3		
Number positive	0	10	1		
Percentage positive	0	29	33		
March:					
Total number of fish	9	43	8		
Number positive	0	2	1		
Percentage positive	0	5	13		
June:					
Total number of fish		25	10	3	3
Number positive		8	7	3	3
Percentage positive		32	60	100	100
July:					
Total number of fish		34	16	5	11
Number positive		4	3	1	6
Percentage positive		13	19	20	55
October:					
Total number of fish		12	37	1	5
Number positive		4	26	1	5
Percentage positive		33	60	100	100
November:					
Total number of fish		11	39	5	
Number positive		2	29	3	
Percentage positive		14	74	60	

[1] Age-group V and older.

Figure 3 is a plot of the seasonal fluctuation of the HM factor with body length. Fish 22 to 25 cm. long were combined in the December sample because the separate groups had few individuals.

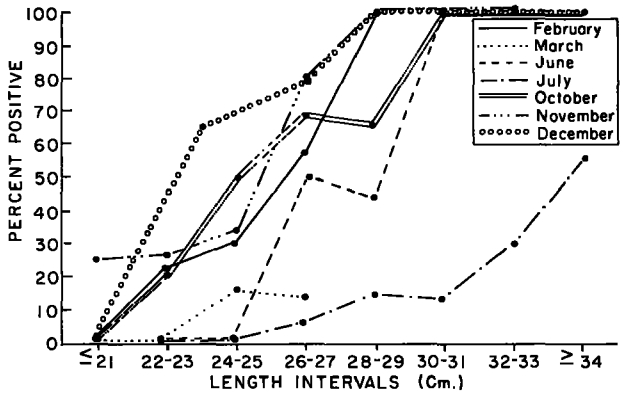

FIGURE 3.—The relation of the frequency of the HM factor to length in female English sole in different months, 1962–63, Port Orchard, Wash.

A distinct seasonal change took place between the spawning in December and the resting stage in July; during this period the HM frequency for a given length interval generally decreased. In the October and November samples, as the ovaries developed for the next spawning season, the HM frequencies in the various length intervals again increased.

Figure 3 does not take into account any growth between the December sample and subsequent collections. If seasonal growth increments were considered, most individuals 26 to 29 cm. long in December would be 30 to 33 cm. long in July. This statement is based on the assumption that El Sayed's (1959) growth estimates for English sole from Holmes Harbor in Northern Puget Sound are applicable to the Port Orchard population. A comparison of the

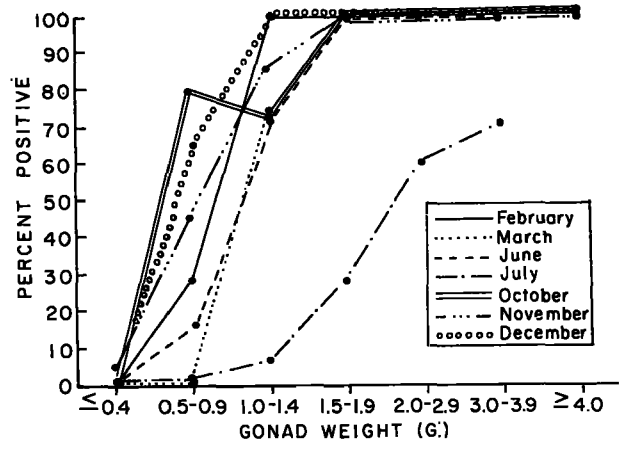

FIGURE 4.—The relation of HM frequency to gonad weights in female English sole, 1962–63, Port Orchard, Wash.

HM frequency in these two length intervals in December with the next higher interval in July still indicates a striking variation of frequency.

Ovarian weight and HM frequency are related (fig. 4). Gonad weight and HM frequency increased simultaneously. A qualitative seasonal variation in the factor's presence is again indicated. All ovaries weighing more than 1 g. that were taken during or immediately following the spawning season were from HM-positive individuals. Even a considerable number of the largest ovaries taken during the resting stage were from HM-negative individuals; HM frequency decreased as the ovarian mass decreased.

The relation between gono-somatic ratio (gonad weight expressed as percentage body weight) and HM frequency is generaly similar to that between gonad weight alone and HM frequency (fig. 5).

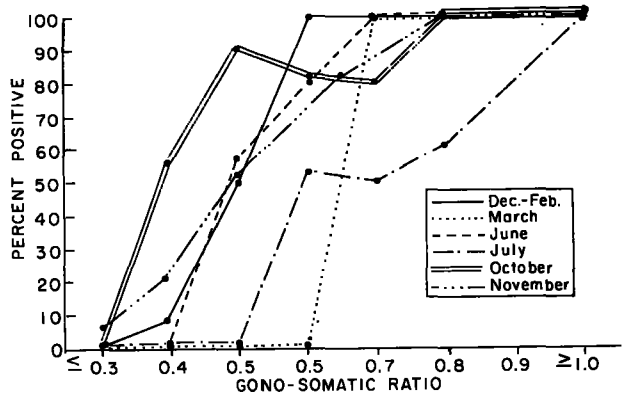

FIGURE 5.—The relation of HM frequency to gono-somatic ratios in female English sole.

The gonad weight shows the relation more clearly than the gono-somatic ratio, however, suggesting that the qualitative seasonal variations of the HM frequency are dependent more on the absolute weight of the gonad than on its mass relative to body weight.

ANALYSIS OF DATA ON HALIBUT

The HM frequency of the various halibut samples is given according to length in table 7. Fish of various ages were combined in the length intervals because of the small samples. The criterion for maturity of halibut ovaries was the presence of macroscopic ova. Personnel of the International Pacific Halibut Commission made all maturity estimates. The HM factor was detected in the serum from all female halibut that were estimated to be mature.

In the sample of June 5, 1962, which gave the best representation of different ages within a given length interval, no indication of an age dependency was detectable. In spite of the small sample sizes, the frequency of the HM factor seems to increase in length in all samples but that of February 23, 1963. This exceptional situation is discussed below.

TABLE 7.—*HM frequencies in female halibut according to length*

Date of collection	Length (cm.)							
	<85		85-100		101-110		>110	
	HM+	HM−	HM+	HM−	HM+	HM−	HM+	HM−
	Number of fish	*Number of fish*	*Number of fish*	*Number of fish*	*Number of fish*	*Number of fish*	*Number of fish*	*Number of fish*
1963								
Feb. 23	2	0	3	1	1	0	0	0
1960								
Apr. 20	0	1	1	3	0	1	7	0
1963								
Apr. 23	0	0	0	2	1	1	1	1
1962								
May 11	0	0	0	2	2	0	2	0
June 5	0	0	6	10	8	8	9	0

Note.—Sampling areas shown in table 2.

As pointed out previously, we detected the HM factor during the spawning season in the serum of certain individual English sole with immature ovaries; apparently a similar condition exists in halibut. Two 76-cm. females taken in the February 23, 1963, sample at the peak of the spawning season were HM positive. [Thompson (1915) defined the spawning season for halibut as extending from December to April with the peak in February.] These fish were smaller than the minimum length reported by Thompson for mature females captured from areas included in this study, and the small ovaries did not give external indications of development. The records of serum titers of the HM-positive females in the sample of February 23, 1963, show that the two HM-positive females which were judged immature had very low titers whereas the mature individuals had titers exceeding a serum dilution of 1/250 (table 8). These findings suggest that a quantitative means may be used to distinguish maturing and mature females from immature females in which the HM factor may also be present.

In the discussion of table 4 in a preceding section, serum titers were compared with qualitative reactions of kidney-tissue fluids from the halibut sample taken on June 6, 1962. The only failures of parallel reactions were in individuals having serum dilution titers of one-eighth or less. This information indicates that kidney-fragment fluids may be useful in researchers' obtaining data relative to maturity and sex by sampling the eviscerated fish in the commercial catch upon arrival in port.

TABLE 8.—*Serum titers and maturity estimates of HM-positive females from halibut sample of February 23, 1963, taken at Cape Flattery, Wash.*

Number of fish	HM serum titer[1]	Estimate of maturity[1]
1	<2	Immature
7	<2	do
2	512	Mature
6	512	do
8	256	do
11	1,024	do

[1] Reciprocal of last positive dilution.

PRODUCTION OF HM FACTOR BY MALES

The link connecting estrogen with the production of serum components related to maturity has been determined by a number of investigators of birds, through the production of these components in males and immature females after artificial stimulation with estrogenic hormones (McDonald and Riddle, 1945; Urist and Schjeide, 1961). Bailey (1957), Urist and Schjeide (1961), and Ho and Vanstone (1961) have likewise demonstrated that artificial stimulation with estrogenic hormones produces a blood serum situation similar to that of the mature female in the teleosts *Carassius auratus*, *Paralabrax clathratus*, and *Oncorhynchus nerka*, respectively. We attempted this procedure in this study with four English sole maintained in an aquarium at the University of Washington College of Fisheries. Each fish was injected intramuscularly with 1 ml. of an aqueous suspension of estrone (5 mg./cc.). Two fish survived the initial handling (table 9). Data for the fish firmly establish the presence of the HM factor as a consequence of introducing the estrogenic hormone. Although a control bleeding was not made for fish No. 1, the rise in titer between the first and second bleeding establishes beyond doubt the effect of the estrone injection.

The HM factor was in low concentration in two male halibut taken during the spawning season. The possibility of contamination cannot be excluded because both were taken immediately following the collection of a sample from an HM-positive female, although precautions were taken to minimize contamination.

TABLE 9.—*Effect of estrone injections on the occurrence of the HM factor in the serum of male English sole*

Fish number and time of bleeding	HM reaction	Titer[1]
No. 1:		
Prior to injection	−[2]	−[2]
48 hours after injection	+	8
144 hours after injection	+	256
No. 2:		
Prior to injection	0	0
48 hours after injection	+	<8
144 hours after injection	+	256

[1] Reciprocal dilution. [2] No control bleeding.

The production of the HM factor in the serum of male English sole after estrogenic stimulations and the natural occurrence of the factor in male halibut during the spawning season indicate that males should be included in investigations of the HM factor. The natural occurrence of the factor in males may have a number of causes. Although the testes of the HM-positive male halibut appeared normal, the presence of the factor may have been due to low-level secretions of estrogenic hormones. Hermaphoditism has been reported in a diverse range of teleosts, including clupeids (Fowler, 1912), salmonids (Ross, Yasutake, and White, 1963), siluroids (Singh and Sathyaneson, 1961), cyprinodonts (Chidester, 1917), centrachids (James, 1946), and scombroids (Uchida, 1961). A further possibility is the stimulation by estrogenic hormones of exogenous origin through ingestion of mature females of smaller species of flatfish or possibly fish from other families. Estrogenic hormones are effective when administrated orally to mammals and presumably could be similarly effective in fish.

BIOLOGICAL IMPLICATIONS OF THE PRESENCE OF THE HM FACTOR

The English sole has been demonstrated to have a qualitative seasonal variation of the HM factor. The factor occurs first during the spawning season, at least as early as the second year in some individuals. After the spawning season through at least midsummer, fewer and fewer individuals retain the factor in the serum. As a new spawning season approaches, the factor gradually reappears.

Both the disappearance and the reappearance of the factor are more pronounced with increase of body length and ovarian mass. This relation may be the result of resorption of residual vitellin retained with the ovary, since the large ovaries retain a greater volume of unspawned ova.

A qualitative seasonal variation was not found in mature halibut, but we lack samples taken later than June. Thompson (1915) reported a continuous development of the ova which are to mature in the succeeding generation in the spent halibut ovary; vitellin synthesis may be a perennial process in the mature female halibut. The detection of serum vitellin in postspawning Atlantic salmon (*Salmo salar*) by Fine and Drilhon (1964) suggests its perennial occurrence in this species.

The presence of the HM factor during spawning season in immature females of both species indicates that such an occurrence may be widespread among the Pleuronectidae. Incomplete maturation preceding initial spawning in the Pleuronectidae has been reported previously. Thompson (1915) stated that contemporary investigators had found some ova in immature pleuronectid females which appeared ready to ripen but which failed to do so because the ovary, as a whole, was not yet ready. Franz (1910) reported finding this condition most marked in plaice during the last winter preceding initial spawning.

PRACTICAL APPLICATIONS OF THE HM FACTOR

As a practical procedure, the determination of the HM factor appears to have its greatest potential value in the larger pleuronectid species. In large species such as halibut or starry flounder, the sex cannot be determined at sight, except in ripe individuals. Small samples of blood taken at the time of tagging could yield information on sex and maturity without endangering the fish. Repeated bleedings of four starry flounders kept in captivity did not appear to endanger these fish. Routine practical applications to smaller species seem less likely. The sexes of smaller flatfish species, such as English sole, are generally evident by external examination; and bleeding English sole, where required in this study, caused high mortality. Evisceration of the commercial halibut catch at sea does not preclude practical application, since analysis of kidney-fragment fluids can be made after the catch arrives in port. On the other hand, smaller species are brought to port in the round and sex information can be obtained directly. Smaller species, however, are frequently more readily available in greater

numbers; they are valuable for clarification of general principles which may be applied to other species as well.

SEPARATION OF MATURE FEMALES FROM OTHER HM-POSITIVE INDIVIDUALS

Separation of HM-positive males and immature females from mature females appears possible by quantitative means. The HM serum levels of mature female halibut taken during the spawning season had titers above 200, whereas the titers of HM-positive males and immature females, which were found only at this time, were less than 2.

An extension of the single diffusion quantitation, as used in this study, may be applied where routine quantitation is required. From figure 2 it can be observed that the diameter of the precipitin ring decreases regularly as the HM concentration decreases. A measurement of the diameter of the precipitin ring formed by the undiluted fish serum could give the approximate titer.

AREAS FOR FURTHER INVESTIGATION

Several areas for further study are evident. More frequent and larger samples are desirable. As indicated above, routine quantitation may be necessary during the spawning season, and a knowledge of the quantitative seasonal fluctuation of the HM factor in a given species would be useful. Perhaps the relative HM concentration can be related to such factors as age, weight, or fecundity. More extensive histological examinations of the ovary would help, and a similar examination of the pituitary gland may establish more fundamental criteria for the occurrence of the factor. A biochemical assay might indicate that the composition of the factor in fishes is related to analogous components in other vertebrates.

THE BROADENING APPLICATION OF SEROLOGY IN FISHERY RESEARCH

Serological techniques have had increasing application in fishery problems during recent years. This research has been directed mainly toward racial studies of serum antigens or red blood cell antigens. Many of the current approaches to serological investigations of populations were discussed in a symposium moderated by Cushing (1962), and the subject has been reviewed recently by Marr and Sprague (1963) and Cushing (1964).

Antigenic differences at species level have also been investigated. Ridgway and Klontz (unpublished data) and Sindermann (1962) have found distinct species-specific antigenic characteristics in red blood cells and serum of species of Pacific salmon and Atlantic clupeiods, respectively. Ridgway (1963) reported species-specific antigens in muscle tissue of certain tuna species, in addition to species-specific blood serum components. This finding offers a possible serological means of distinguishing larvae of these species.

We hope that this study will help broaden the interest in application of serological methods to other areas of fishery biology. Because components similar to the HM factor have been detected in a diverse range of teleosts, a similar approach presumably could be used throughout this class of vertebrates. We feel that this approach can be a valuable supplement to investigating maturity in fish, though perhaps not universally applicable.

SUMMARY

A serological investigation of a serum vitellin factor in mature and maturing female flatfish was made on English sole and Pacific halibut. Immunodiffusion techniques with antisera prepared in rabbits stimulated with egg vitellin extracted from starry flounder eggs were used to detect the factor. In English sole the factor's occurrence was compared with age, length, gonad weight, and gono-somatic ratio. A qualitative seasonal variation was found; individuals with heavier ovaries during the summer were more likely to retain the factor in the serum. The presence of the factor in female halibut was compared with length, age, and maturity. A qualitative seasonal variation in mature halibut could not be studied because no samples were available during the summer or autumn. The factor was found in some immature females of both species during their spawning seasons. Production of the factor in male English sole by injections of estrogenic hormones associates synthesis of the factor in females with production of estrogen. Determination of the factor appears to have potential value as a supplement to other means of investigating maturity, particularly in large species.

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