stearate and longer chain saturated fatty acids caused clumping of platelets with release of serotonin and histamine in rabbit platelet-rich plasma. Release of ADP by fatty acids by direct damage to the platelet membrane, as well as activation of adsorbed Hageman factor, has been postulated to cause platelet aggregation(8). The differences in behavior observed in this study between long chain saturated fatty acids on the one hand, and the short chain fatty acids and the unsaturated fatty acids, linoleic and linolenic, on the other, in their effects on platelet aggregation need further explanation. Owren *et al*(9) found that an increased thrombotic tendency in patients is associated with an increased activity of the anti-Willebrand factor and factor VIII. It may be that the unsaturated fatty acids prevent platelet aggregation by lowering the activity of the anti-Willebrand factor. Whereas Owren *et al*(9) reported that only linolenic acid was effective in reducing platelet adhesiveness in humans with cardiovascular disease, our *in vitro* studies with pig platelets have shown that linoleic is also capable of retarding platelet aggregation.

Although long chain fatty acids injected into dogs were thrombogenic and fatal according to some investigations, they were reported to be inactive when incubated with albumin(6). This suggests that under certain conditions plasma-free fatty acids may not be fully or effectively bound by albumin. In the present study the fatty acid dispersions were made in saline. Whether solubilization of the fatty acids using albumin and other solubilizers will affect their observed effects on platelet aggregation needs further study.

Mechanisms by which linoleic and linolenic acids may bring about inhibition of platelet aggregation alone or in the presence of long chain saturated fatty acids might be either by preventing damage to the platelet membrane, or by lowering the activity of a factor or factors which promote aggregation.

1. Shermer, R. W., Mason, R. G., Wagner, R. H., Brinkhous, K. M., J. Exp. Med., 1961, v114, 905.
2. Mitchell, J. R. A., Sharp, A. A., Brit. J. Haematol., 1964, v10, 78.
3. O'Brien, J. R., J. Clin. Path., 1962, v15, 446.
4. Gaarder, A., Jonsen, J., Laland, S., Hellem, A., Owren, R. A., Nature, 1961, v192, 531.
5. Connor, W. E., Poole, J. C. F., Quart. J. Exp. Physiol., 1961, v46, 1.
6. Connor, W. E., Hoak, J. C., Warner, E. D., J. Clin. Invest., 1963, v42, 860.
7. Day, H. J., Soloff, L. A., Clin. Res., 1963, v11, 192.
8. Haslam, R. J., Nature, 1964, v202, 765.
9. Owren, P. A., Hellem, A. J., Ödegaard, A., Lancet, 1964, ii, 975.
10. Born, G. V. R., Cross, M. J., J. Physiol., 1963, v168, 178.
11. Mustard, J. F., Murphy, E. A., Roswell, H. C., Downie, H. G., Am. J. Med. Sci., 1962, v33, 621.
12. Soloff, L. A., Wideman, M. P., Nature, 1963, v199, 695.
13. Stigter, D., Overbeek, J. T. G., quoted by Reerink, H., J. Colloid Sci., 1965, v20, 217.
14. Shore, P. A., Alpers, H. S., Fed. Proc., 1963, v22, 504.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Free Plasma Estrogens in the Channel Catfish.*† (30703)

B. E. ELEFTHERIOU, K. W. BOEHLKE AND O. W. TIEMEIER
(Introduced by M. X. Zarrow)
Department of Zoology, Kansas State University, Manhattan, Kan.

Recent interest has been aroused in isolation, identification and measurement of estrogenic steroids in various fish. Estradiol-17β, estrone and estriol have been identified and measured in ovaries and blood of several spe-

* Contribution No. 353 from Agri. Exp. Station and No. 380 from Dept. of Zoology.
† Supported in part by grant 2R01-HD-00013 from Nat. Advisory Child Health and Human Development Council, Nat. Inst. Health.

cies of fish before and during the spawning period(1-12).

Heretofore, however, no attempt has been made to measure levels of free plasma estrogens at different times before spawning and as a function of ovarian weight. In the present study identification has been made of ovarian and plasma estrogens in the Channel Catfish, *Ictalurus punctatus*. In addition, free plasma estrogens were measured in several Channel Catfish with different ovarian weights and at different times before spawning.

Materials and methods. Female catfish weighing 681-1680 g were killed and blood collected by cardiac punctures. Plasma was obtained by centrifugation of whole blood at 1000 $\times$ *g* for 10 minutes. The plasma then was frozen for future analysis. The ovaries were excised, weighed to the nearest tenth of a gram and frozen for future estrogen identification.

Extraction of the estrogens was made using a modification of the technique described by Veenhuizen *et al*(13). The total ovary weight (ovary plus ova) from 9 catfish was 684.2 g. The ovaries were homogenized in 1.5 liters of distilled water in a Waring blendor. The homogenate was extracted 2 times with 4 volumes of ether. The combined ether extracts were taken to dryness *in vacuo* under nitrogen at 40°C. The residue was dissolved in 100 ml of toluene and washed 3 times with an equal volume of 5% NaOH. The NaOH fractions were pooled and backwashed with 50 ml of toluene to insure removal of all fats. The pH of the NaOH fraction was lowered to 7-9 with 6 N H_2SO_4 and neutralized fraction was then extracted 3 times with an equal volume of benzene. The extract was taken to dryness *in vacuo* under nitrogen at 40°C.

The estrogens were separated and identified by using thin-layer chromatography on silica gel G (Merck, Germany). After a portion of the estrogen extract was spotted, the plates were run 2-dimensionally in ethyl acetate-hexane (1:1). Standard estrogens were spotted on a similar plate and run along with the unknown sample. The plates were developed by spraying with a solution of 10% phosphomolybdic acid in absolute ethanol and heating them at 110°C for 20 minutes. The Rf values of estrogens run 2-dimensionally in this system were found to be: Estriol-0.061 $\pm$ .013, epiestriol-0.231 $\pm$ .036, 16-ketoestradiol-0.429 $\pm$ .058, estradiol-17β-0.572 $\pm$ .065, estradiol-17α-0.639 $\pm$ .059 and estrone-0.733 $\pm$ .062 in the first direction. In the second direction the values obtained were: Estriol-0.059 $\pm$ .012, epiestriol-0.195 $\pm$ .026, 16-ketoestradiol-0.362 $\pm$ .044, estradiol-17β-0.507 $\pm$ .039, estradiol-17α-0.581 $\pm$ .046 and estrone-0.707 $\pm$ .060. After the estrogens were initially identified, they were isolated and acetylated along with known estrogen standards and run in one direction on silica gel G thin-layer chromatography plates. Identification was also made by U.V. absorption(14).

When identification was complete, the plasma samples were analyzed to measure quantitatively the free plasma estrogens. Known amounts of labeled C^{14}-4-estrone and C^{14}-4-estradiol-17β were each added to the plasma samples in order to measure the per cent recovery after the extraction procedure. Plasma samples were extracted in a similar manner to that used for the ovaries except that smaller volumes of the various solvents were used. The samples were extracted 3 times with equal volumes of ether, and the residue after drying was dissolved in 10 ml of toluene rather than 100 ml. The NaOH fraction was backwashed with 5 ml rather than 50 ml of toluene. After the benzene fraction was dried, the extract was dissolved in 0.1 ml of chloroform-methanol (1:1) and spotted on a silica gel G thin-layer chromatography plate. The samples were run 2-dimensionally in ethyl acetate-hexane (1:1) along with standard estrogens. After the run was completed, the areas containing the various estrogens were scraped off the plate individually and extracted 2 times with 3 ml of chloroform-methanol (1:1). After the chloroform-methanol was dried, the estrone and estradiol-17β samples were dissolved in 1 ml of absolute ethanol from which a 0.1 ml aliquot was taken. This aliquot was added to 10 ml of scintillation fluid and counted in a Packard Tri-Carb model 3314 Liquid Scin-

tillation Counter to measure recovery. The tubes containing estrone and estradiol-17β then were taken to dryness. Fluorescent measurements were made by a modification of the method described by McAnally *et al* (15). The estrogens in all tubes were dissolved in 0.1 ml of absolute ethanol to which 0.7 ml of 90% H_2SO_4 was added. The tubes were shaken and then incubated for 20 min at 80°C. The samples then were cooled and 4.3 ml of 65% H_2SO_4 added. Fluorescent readings were made in a Turner model 110 Fluorometer with a Kodak 47B and Kodak 2A for primary filters and a Kodak 2A-12 plus a 10% neutral density filter for the secondary filters. The values obtained were extrapolated from a standard curve and corrected to 100 ml of plasma and 100% recovery. The recovery was found to range from 28.59% to 84.87%, average 65.04%. These values were then plotted against weight of ovaries (Fig. 1). Each value is the mean of 2-3 plasma samples.

Results and discussion. Thin-layer chromatography in ethyl acetate:hexane (1:1) solvent system, U.V. absorption and acetylation of unknown and standard estrogens established that the plasma and ovarian estrogens in the catfish are estradiol-17α, estradiol-17β, estrone, estriol, epiestriol and 16-ketoestradiol. These 6 estrogens must account for the entire estrogenic pool of the catfish at 1-3 weeks before spawning.

Results indicate that as the ovary increases in weight from approximately 14 g to 100 g, all the free plasma estrogens also increase proportionally. Thus, in this species, the increase in weight of the ovary may reflect an increased production and release of estrogens from the ovary, although the possibility exists that there may be a slower liver inactivation for which the present experiment did not analyze.

In order of decreasing concentration the estrogens are: ketoestradiol, estriol, epiestriol, estradiol-17β, estrone and estradiol-17α. At the lowest ovarian weight, free plasma estrogens range from a low of 1.53 μg/100 ml for estradiol-17α to a high of 5.99 μg/100 ml of plasma for 16-ketoestradiol (Fig. 1). At the highest ovarian weight, estrogens vary from

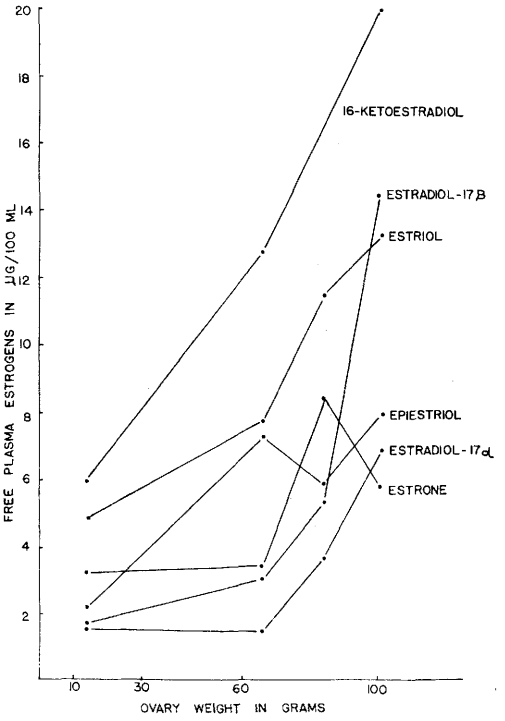

FIG. 1. Free plasma estrogen level (μg/100 ml) in Channel Catfish with different ovarian weight 1-3 weeks before spawning.

a low of 5.72 μg/100 ml for estrone to 19.97 μg/100 ml for 16-ketoestradiol. In general, as the ovary increases in weight, there is a 2-fold increase in estrone and an 8-fold increase in estradiol-17β, while the other estrogens exhibit a 3-fold increase. This compares with a 6-fold increase at time of spawning for the Atlantic Salmon(12). However, in the Atlantic Salmon, estrogens reached a maximum of 7 μg/100 ml blood while at the highest ovarian weight, just before spawning, of the present study total free estrogen fraction reached a combined high of 66.20 μg/100 ml of plasma from a low of 18.81 μg/100 ml or a 3-fold increase.

Some of the waning which occurs for epiestriol, estrone, and to a minor extent for estradiol-17α, may reflect more active utilization and removal from free fraction for these particular hormones at different times, or possibly some error in analysis.

Summary. Isolation of free plasma estrogens from blood and ovaries of the Channel Catfish, *Ictalurus punctatus*, led to identifi-

cation of 16-ketoestradiol, estriol, epiestriol, estrone, estradiol-17α and estradiol-17β. It was found that as the ovary increases in weight at 1-3 weeks before spawning, there is a gradual increase for all free plasma estrogens. In order of their increasing concentration, in μg/100 ml of plasma, the free plasma estrogens were found to be: estradiol-17α, estrone, estradiol-17β, epiestriol, estriol and 16-ketoestradiol.

1. Boticelli, C. R., Hisaw, F. L., Jr., Roth, W. D., Proc. Soc. Exp. Biol. and Med., 1963, v114, 255.

2. Wotiz, H. H., Boticelli, C., Hisaw, F. L., Jr., Ringler, I., J. Biol. Chem., 1958, v231, 589.

3. Wotiz, H. H., Boticelli, C. R., Hisaw, F. L., Jr., Olsen, A. G., Proc. Nat. Acad. Sci. U. S., 1960, v46, 580.

4. Simpson, T. H., Wright, R. S., Hunt, S. V., J. Endocrinol., 1963, v26, 499.

5. Chieffi, G., Gen. Comp. Endocrinol., Suppl., 1962, v1, 275.

6. Hisaw, F. L., Jr., Proc. 22nd Ann. Biol. Colloq., Physiol. Reprod., Oregon State Univ. Press, Corvallis, 1963, 1-16.

7. Dean, F. D., Jones, I. C., J. Endocrinol., 1959, v18, 366.

8. Gottfried, H., Hunt, S. V., Simpson, T. H., Wright, R. S., ibid., 1962, v24, 425.

9. Lupo, C., Chieffi, G., Nature, 1963, v197, 596.

10. Gottfried, H., Steroids, 1964, v3, 219.

11. Cedard, L., Nomura, T., Bull. Inst. Oceanogr., 1961, v1196, 1.

12. Cedard, L., Fontaine, M., Nomura, T., Comp. Rend. Acad. Sci., 1961, v252, 2656.

13. Veenhuizen, E. L., Erb, R. E., Gorski, J., J. Dairy Sci., 1960, v43, 270.

14. Zaffaroni, A., Rec. Prog. Horm. Res., 1953, v8, 51.

15. McAnally, J. S., Hausman, E. R., J. Lab. Clin. Med., 1954, v44, 647.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Free Plasma Estrogens in the Deer.*† (30704)

B. E. ELEFTHERIOU, K. W. BOEHLKE, A. ZOLOVICK AND F. KNOWLTON
(Introduced by M. X. Zarrow)
Department of Zoology, Kansas State University, Manhattan, Kan.

Recent advances in the separation and quantitative determination of plasma estrogens have resulted in isolation and characterization of several estrogenic steroids in various species. Heretofore, however, concentration of plasma estrogens during menstrual cycle and pregnancy have been measured only in the human(1-5).

The present study was undertaken to identify and measure levels of free plasma estrogens during estrus and pregnancy in the white-tailed Texas deer, *Odocoilus virginianus texanus*.

Materials and methods. Blood was collected from 25 adult female white-tailed Texas deer during estrus and at stages of pregnancy ranging from 6 to 23 weeks. Number of samples collected for estrus were 3, for 6 to 8 weeks of pregnancy 6, for 11 to 14 weeks 5, for 15 to 18 weeks 7 and for 20 to 23 weeks 4. The samples were collected in the field at the wildlife game preserve in Sinton, Texas. Plasma was obtained by centrifugation and frozen for later analysis.

Initial identification of the free plasma estrogens was made on six 300 ml samples of plasma. Extraction of the estrogens was made using a modification of the technique described by Veenhuizen *et al*(6). The plasma was extracted 3 times with an equal volume of ether. The extracts were pooled and dried *in vacuo* under nitrogen at 40°C. The residue was taken up in 10 ml of toluene and washed 3 times with 5 ml aliquots of 5% NaOH. The pooled NaOH fractions then were backwashed with 5 ml of toluene. The pH of the NaOH fraction was lowered to 7-9 with 6 N H_2SO_4. This neutralized fraction

* Contribution No. 355 from Dept of Zoology, Kansas Agri. Exp. Station and No. 381 from Dept. of Zoology, Manhattan.

† Supported in part by grant 1R01-HD-00013 from the National Advisory Child Health and Human Development Council, N.I.H., Bethesda, Md.