Environmental estrogens, which exert effects similar to natural endogenous estrogens, can cause reproductive disturbances in male fish [8, 18, 37, 39]. Abnormalities have been observed in male carp testes exposed experimentally to estrogenic chemicals, including hermaphroditism [12, 13], progressive reduction in spermatozoa and reduced seminiferous tubules [14]. In addition, we have reported that the testes of wild carp from a river contaminated with environmental estrogens is significantly reduced in weight [17]. The pituitary gonadotropin controls the function of the testis, but no data are available about the effects of estrogenic chemicals on the gonadotropes.

Identification and distribution of gonadotropes of teleost fish have been established by immunocytochemical techniques using antisera against mammalian gonadotropins [7] or teleost gonadotropins [3, 5, 10, 21, 26, 34, 36]. Little have been published on the immunocytochemistry of the gonadotropes of the common carp, *Cyprinus carpio* [1]. The present study examines the adverse effect of the estrogenic chemicals on the gonadotropes in the adenohypophysis of the common carp *Cyprinus carpio* during the annual reproductive cycle, using immunocytochemical and morphometric methods.

**MATERIALS AND METHODS**

**Fish:** Of 115 adult male carp, *Cyprinus carpio*, collected from June 1998 to March 2001 and used in the study of testes [17], 45 were selected at random for the present study. The fish were caught in the Ishizu and Wada Rivers at Sakai City in Osaka. The sampling site on the Ishizu River was contaminated by sewage treatment works and industrial effluents as well as agricultural runoff. Water samples from the sampling sites on the Ishizu and Wada Rivers were chemically analyzed for the presence of three estrogenic chemicals, nonylphenol, bisphenol A and 17β-estradiol [17]. Control fish were obtained from a pond at Agriculture, Food and Environmental Sciences, Research Center of Osaka Prefecture and were fed with commercial fish food (Nippon Formula Feed Mfg., Osaka).

**Tissue processing:** Immediately after decapitation of the fish, the pituitary gland was gently separated from the *Sella turcica* and fixed in 10% neutral formalin for 24 hr, dehydrated in a graded series of ethanol solutions, and embedded in Tissue Prep (Fisher Scientific, Pittsburgh, PA, U.S.A.). Serial 5 µm sagittal sections were cut and mounted on slides coated with poly-1-lysine (50 µg/ml). The sections were immunocytochemically stained using the avidin-biotin-peroxidase complex (ABC) method. Immunolabelling was

**ABSTRACT.** The adverse effect of estrogenic chemicals on luteinizing hormone-immunoreactive (LH-ir) cells in the adenohypophysis of common carp (*Cyprinus carpio*) was examined using immunocytochemical and morphometric methods. Adult male fish were collected from two contaminated sites (Ishizu and Wada Rivers) and from a control pond at Agriculture, Food and Environmental Sciences, Research Center of Osaka Prefecture. The concentration of nonylphenol, bisphenol A and 17β-estradiol in the Ishizu River was 3–4 times higher than that in the Wada River. The proportion and size of LH-ir cells were evaluated using the point-counting method by optical microscopy. In control carp, the proportion of LH-ir cells in the breeding season was significantly lower than in the pre- and post-breeding seasons. The same tendency was also found in Ishizu and Wada River carp, but without statistical significance. The proportion of LH-ir cells in Ishizu River carp was significantly lower than that of the control and the Wada River in all seasons. The LH-ir cells in control carp increased in size in the breeding season. LH-ir cells in Ishizu River carp were significantly (p<0.05) smaller than those in control fish, but not different from Wada River carp. A disturbance in the secretory function of LH-ir cells was found in carp from the Ishizu River; granulation and vacuolation were not in synchronization with those of control and Wada River fish. Our data suggest that the estrogenic chemicals in the Ishizu River interfere with functions of LH-ir cells directly or through the testis.
performed using a rabbit antiserum raised against human luteinizing hormone (hLH) β-subunit [1:5,000 dilution; National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), CA, U.S.A.]. The sections were treated with the antiserum overnight, washed in 0.01 M phosphate-buffered saline (PBS, pH 7.4), incubated with anti-rabbit second antibody (Vectastain, Vector Laboratory, Burlingame, CA, U.S.A.), and washed in PBS. They were then incubated with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.005% H2O2 for about 10 min, counter-stained with hematoxylin, and then observed with an optical microscope. The specificity of the antiserum was verified by absorption tests and by substitution of normal rabbit serum for the specific antiserum. Adjacent sections were stained with periodic acid Schiff (PAS).

*Morphometry:* Optical colour micrographs (23 ± 3 sheets/fish) were taken from various locations of the sections through the adenohypophysial proximal pars distalis (PPD). The cell area and the proportion of LH-ir cells were determined by a point-counting method using a sheet of cellophane, with dots at 1 cm intervals in perpendicular directions, which was superimposed over the photomicrograph (Fig. 1). The number of points over the nucleus of the LH-ir cells, as well as negative cells in PPD, was determined. At this magnification (× 1,200), a nucleus could not correspond to more than one point. The percentage of LH-ir cells was calculated as the number of LH-ir cells × 100/total number of counting cells (1,060 ± 22 per fish). The area of the LH-ir cells was measured by counting the number of points over each immunoreactive cell, regardless of the presence or absence of the nucleus. The total number of points corresponding to LH-ir cells was divided by the number of nuclei of the LH-ir cells, and the resulting value was used as an index representing the size of the LH-ir cells.

*Statistical analysis:* Results were expressed as mean ± SE. To analyze the results, ANOVA with Fisher’s least significant differences test was used. Differences at p<0.05 were considered significant.
RESULTS

Morphology and immunocytochemistry of LH-ir cells:
The carp pituitary consists of the adenohypophysis (AH) and the neurohypophysis (NH). The AH is further divided into three regions: rostral pars distalis (RPD), PPD, and the pars intermedia (PI). From the dorso-caudal portion of the gland, the NH deeply invades the AH and sends its complicated ramifications into the PI (Fig. 2). LH-ir cells exhibiting strong affinity with PAS are distributed throughout the PPD, forming small clusters or strands. These cells are large polygonal or round in shape, and often contain coarse secretory granules and an ovoid, eccentrically located nucleus. During the annual reproductive cycle, LH-ir cells in control fish and Wada fish showed several forms of granulation and degranulation; after the most extensive degranulation the cells included large vacuoles and eccentrically located flat or pycnotic nuclei. In the pre-breeding season, the LH-ir cells showed a gradual accumulation of secretory granules (Fig. 3a). The LH-ir cells underwent hypertrophy, with more granular accumulation at the beginning of the breeding season. During spawning time (the breeding season) several LH-ir cells displayed partial degranulation and vacuolation. In the late spawning and post-breeding season, a large number of LH-ir cells were completely degranulated and were occupied by large vacuoles; the vacuolated cells were more irregular in shape than the granulated ones (Fig. 3b). In Ishizu River fish the LH-ir cells showed the same reaction with the antiserum and the same distribution in the PPD; however, vacuoles appeared in unexpected season (2 fish out of 5) in the pre-breeding season (Table 1).

Morphometrical results: Table 2 shows the proportion and area of LH-ir cells in our carp during the annual reproductive cycle. The percentage of LH-ir cells showed a significant decrease in the breeding season of the control carp relative to values in the pre- and post-breeding seasons. The same tendency was seen in LH-ir cells from the Ishizu and Wada carp, although not significantly. The percentage in Ishizu River carp was significantly lower than that of the control and Wada River carp.

The size of the LH-ir cells was significantly larger in the breeding season than in the pre-breeding season in control carp. This annual cyclic change was not found in Ishizu and Wada Rivers carp. The LH-ir cells of Ishizu River carp were significantly (p<0.05) smaller than those of control fish and the same as for Wada River carp.

DISCUSSION

Pituitary gonadotropins (GTHs), follicle-stimulating hormone (FSH) and LH are the key hormones involved in reproduction in vertebrates, regulating gonadal gametogen-
Secretion of gonadotropins from the pituitary is controlled by gonadotropin-releasing hormone (GnRH) together with steroid feedback mechanisms. GnRH stimulates LH release; however, the response of the gonadotropin to GnRH in the common carp was found to be different, and to depend on the gender and the phase of sexual ontog-
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eny [20]. Positive and negative feedback mechanisms of sex steroid jointly act concurrently to regulate GTH-II release in adult teleosts. The principal site of positive feedback is the GTH-II cell, in which testosterone and estradiol mediate GnRH-stimulated GTH-II release [19]. Steroids operate by binding to specific receptors; specifically, estrogen receptors have been found in gonadotropes [19, 45]. Estrogenic chemicals can bind to the estrogen receptors and so to induce or attenuate estrogen-dependent biological processes [27]. Of many estrogenic chemicals tested, bisphenol A and alkylphenolic compounds showed the greatest affinity for the carp estrogen receptor [23]. Nonylphenol is known to inhibit testicular growth in developing rainbow trout [18]. There is an evidence that the estrogenic effects of nonylphenol in fish are mediated by interaction with the estrogen receptors [48].

Our morphometric data on LH-ir cells from the Ishizu carp revealed a disturbance in the secretory functions: granulation and vacuolation were not in synchronization with those of control and Wada River carp. Furthermore, LH-ir cells from Ishizu River carp showed significantly lower cell proportion than those in other sites. These phenomena might be a drop of the function of LH-ir cells. As the estrogenic chemicals in the Ishizu River adversely affect the testis development of the carp [17], they may interfere with the function of LH-ir cells directly or via testis.

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REFERENCES


