Cytoplasmic membranes of these large cells spread widely, showing an intricate fine texture (Fig. 2*B*). After 3 to 4 days there appeared in the Golgi region granules which gradually increased in size and number. After 6 days, bacteria, probably symbionts of the embryo, were seen engulfed in the cytoplasm. No cell divisions were observed. Although the origin and nature of the large cells remain obscure, they resemble phagocytes obtained from the cultured circulatory organ of Lepidoptera (*13*). Fibroblast-like cells and epithelial cells of both smaller and larger tissue fragments in the stage of blastokinetic movement also became attached to the glass surface and grew in vitro. After 24 hours of initial cultivation, fibroblast-like cells appeared and continued to grow, forming networks (Fig. 2, *C* and *D*). After 48 hours of cultivation, epithelial cells began to grow, forming a fairly compact cell sheet (Fig. 2, *E* and *F*). Cells derived from smaller tissue fragments began to degenerate and eventually became detached from the glass surface on the 15th day. Cells derived from larger tissue fragments, which had contractile movements for more than 15 days, continued to grow for more than 40 days.

Whether the need for fairly large numbers (100 embryos) for each successful cultivation test reflected a statistical chance to find in this number an adequate supply of tissues at the proper stage of development, or whether there was a definite need for some growth factors supplied in adequate amounts only by larger tissue masses, could not be ascertained. The latter seems more plausible at the moment. It also would explain the better growth consistently obtained from larger fragments of tissues compared to poor growth from smaller fragments. So far, it has not been possible to determine whether cells that became attached to the cover glass within the initial 2 hours of cultivation, and that degenerated usually after 8 days, were derived from the same tissues as cells that eventually grew well, or whether these earliest growing cells were hemocytes of the embryo.

The medium for cultivation cannot be considered as an "insect tissue culture medium," since differences between groups of arthropods are often more pronounced than differences between mammals on one side, and birds or reptiles on the other. Several attempts have been made to improve the composition of the culture medium. Among others, hemolymph of Crustacea was tried as an additive to our medium, but it failed to enhance the growth of cells in vitro.

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Carotenoids of Cavernicolous Crayfish

Abstract. *Small amounts of β-carotene and lutein were found in* Orconectes pellucidus pellucidus. Cambarus bartonii tenebrosus *from the same cave contained much less carotenoid than surface crayfish. Astaxanthin, the principal carotenoid of most Crustacea, was absent from* O. p. pellucidus, *but accounted for 83 percent of the carotenoid of* C. b. tenebrosus. *These findings support other observations that pigmentation is dependent on the amount of carotenoid in the diet rather than on the presence of light. Furthermore, they suggest that* O. p. pellucidus *has lost or has never developed the ability to oxidize dietary carotenoids.*

Crustacea contain large amounts of carotenoid, most of it in the form of astaxanthin, which usually occurs conjugated with protein in the integumentary pigment of the animals. This is true of surface forms as well as bathypelagic and benthic members of this class. Astaxanthin is formed by the oxidation of dietary carotenoids (*1*). Intermediate products of oxidation have been demonstrated in *Artemia salina* (*2*) and *Carcinas maenas* (*3*). Vitamin A, also an oxidation product of dietary carotenoids, is involved in the visual process of many species of Crustacea including the crayfish *Orconectes virilis* and *Procambarus clarkii* (*4*). Vitamin A has not been detected, however, in many other crustacean species (*5*).

Cavernicolous Crustacea exhibit very little external pigmentation and their eyes are degenerate or absent. No carotenoid was found in the isopod *Aselus aquaticus cavernicolous* (*6*) or in cavernicolous amphipods of the genus *Niphargus* (*7*). Beatty (*8*) examined cave detritus and found substantial amounts of carotenoid in flood debris consisting chiefly of old leaves and pieces of wood. He concluded that the absence of pigment might result from the absence of light (*7*). The validity of this hypothesis was questioned by Maguire (*9*), who showed that color development in the crayfish *Procambarus simulans simulans* was independent of light and dependent upon nutrition. The presence of carotenoids in benthic marine Crustacea, living in the perpetual absence of light, tends to confirm that carotenoid pigmentation is not light induced.

We had the opportunity to compare

Table 1. Carotenoid content of *Orconectes pellucidus pellucidus* and *Cambarus bartonii tenebrosus*.

Species	Weight (g)		Total carotenoid (μg)
	Wet	Oil	
O. p. pellucidus	65.6	0.774	15.7*
C. b. tenebrosus	127	1.27	722

* Since the spectrum obtained for the total extract exhibited much nonspecific absorption (Fig. 1), this value represents the sum of the fractionated carotenoids.

Table 2. Carotenoid composition of *Orconectes pellucidus pellucidus* and *Cambarus bartonii tenebrosus*.

Probable identity*	Absorption maxima (hexane) (mμ)	Carotenoid (%)†	
		O. p. pellucidus	*C. b. tenebrosus*
β-Carotene (E)	477, 449, 425	41.6	6.02
Xanthophyll ester‡	475, 448, 425		0.89
Astaxanthin ester (E)	467		66.4
Keto-carotenoid	442		3.36
Lutein (H)	470, 443, 419	58.4	4.55
Fraction 5	472, 448, 425		2.23
Astaxanthin (H)	467		6.48
Astacene (E-H)§	470		10.1

* Carotenoids are listed in order of elution from alumina column. Partition behavior between hexane and 90 percent methanol is indicated in parentheses: (E), epiphasic; (H), hypophasic. † Carotenoid concentrations calculated as the percentage of total carotenoid estimated as β-carotene. ‡ Absorption spectra were diffuse; the compound may have been cryptoxanthin ester. § An artifact apparently formed during isolation or on column during chromatography.

white eyeless cavernicolous crayfish (*Orconectes pellucidus pellucidus*) with *Cambarus bartonii tenebrosus,* a crayfish not so well adapted for cave life (*10*). This latter species, although generally found in caves, has been reported to be successful also in an epigeal environment (*11*). Twenty-seven adult *O. p. pellucidus* and six adult *C. b. tenebrosus* were collected from a small cave near Cave City, Kentucky. The animals were maintained for 5 days at 4°C in the dark in order to clear exogenous carotenoid from the gut. The whole animals were homogenized with acetone and extracted with acetone three times. The combined extracts were reduced in volume by evaporation under vacuum, diluted with water, and extracted with hexane. The hexane solution was dried over anhydrous sodium sulfate, the solvent removed under reduced pressure after flushing with nitrogen, and the oil weighed (Table 1). A hexane solution of the *O. p. pellucidus* extract was faint yellow, while that of the *C. b. tenebrosus* was bright red. The absorption spectra of the extracts in hexane (Fig. 1) were determined (*12*) and the carotenoid contents estimated as β-carotene (extinction coefficient of 2500 at 449 mμ). Since astaxanthin is the major pigment in *C. b. tenebrosus,* some error is introduced by this method (*13*).

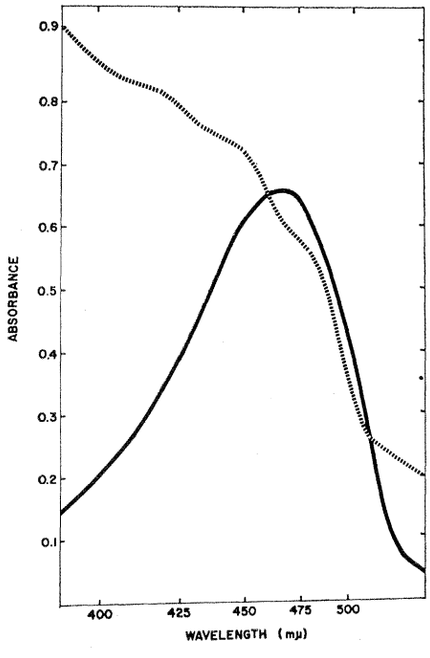

Fig. 1. Absorption spectra of *Orconectes pellucidus pellucidus* (dashed curve) and *Cambarus bartonii tenebrosus* (solid curve) extracts in hexane. Concentrations were 77.4 and 4.23 mg/ml, respectively.

The extracts were chromatographed on columns of neutral alumina (Woelm) "weakened" by the addition of 5 percent water by weight, and eluted by increasing amounts of acetone in hexane. Astaxanthin was eluted by acetone : methanol : glacial acetic acid (90 : 10 : 1, vol/vol), while astacene was eluted by 15 percent KOH in 90 percent methanol. Astacene is an artifact produced by the oxidation of astaxanthin in the presence of alkali (*14*). Treatment with methanolic KOH would therefore generate astacene from any astaxanthin isomer still adsorbed on the column. Two pigments were separated from the *O. p. pellucidus* extract. Eight distinct carotenoids were separated from the *C. b. tenebrosus* extract. Carotenoid identities were assigned on the basis of spectral characteristics and chromatography (Table 2). The partition behavior of β-carotene was epiphasic; that of lutein was hypophasic. The keto-carotenoid may be similar to the carotenoid reported by de Nicola (*15*) and Lenel (*3*).

All fractions were assayed for vitamin A by the Carr-Price reaction. No vitamin A was detected in *O. p. pellucidus* or *C. b. tenebrosus*; however, it is difficult to demonstrate the complete absence of trace amounts of vitamin A by standard procedures (*16*). A small transient peak at 660 mμ was noted in *C. b. tenebrosus* (astaxanthin ester fraction) when tested for vitamin A, and may indicate the presence of retinene$_1$. A positive identification was not possible with this trace component.

The carotenoids present in the diets of these cavernicolous crayfish are probably β-carotene and lutein, since both are found in *O. p. pellucidus.* An evolutionary loss of, or the failure to develop, the ability to generate keto-carotenoids from the conventional plant carotenoids of the diet is suggested by the absence of astaxanthin in this crayfish.

The carotenoid composition of *C. b. tenebrosus* resembles that of other Crustacea (*3*); however, the total carotenoid content of this hypogeal crayfish (568 μg/g of oil) is much lower than that of a typical epigeal crayfish *Orconectes rusticus* (9000 μg/g of oil). This difference probably reflects a greatly reduced carotenoid intake. Fox (17) reported that the spiny lobster *Panulirus interruptus* lost up to 95.5 percent of its original carotenoid when placed on a carotenoid-deficient diet, much of the carotenoid being lost with each molt. A loss of visual function may also occur as a result of decreased provitamin A intake, although many Crustacea possessing functional eyes which contain astaxanthin apparently lack vitamin A (*5*). The *C. b. tenebrosus* did not react when approached by a hand, although epigeal forms assume a defensive stance with their chelae spread wide.

Since vitamin A is absent or greatly diminished in both *O. p. pellucidus* and *C. b. tenebrosus,* this vitamin may serve only a visual function in Crustacea. Fox (*17*) has suggested that astaxanthin may act in the maintenance of crustacean integumentary surfaces and in the secretion of calcareous or mucous materials. The complete absence of astaxanthin in *O. p. pellucidus* suggests that this idea requires further exploration.

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Estrous Synchrony in Mice: Alteration by Exposure to Male Urine

Abstract. *Exposure of grouped, virgin female laboratory mice to urine from male mice for 2 days prior to pairing significantly altered the expected pattern of estrous synchrony. A higher proportion of mice exposed to male urine attained estrus during the 2 days after pairing than did mice exposed to female urine or control mice.*

Female laboratory mice that have been housed in groups show a nonrandom distribution of copulations after individual pairing with a male. Specifically, a higher proportion than expected achieve estrus on the 3rd night after pairing, and a lower proportion on nights 1, 2, and 4. This synchrony of estrus is apparently mediated by olfactory stimuli derived from the male since (i) exposure of the female to a male for 2 days without physical contact results in a higher proportion of copulations on the first night after pairing (that is, the 3rd night after individual exposure to a male); (ii) females which have had their olfactory bulbs removed do not show estrous synchrony; and (iii) exposure of females to cages recently soiled by males slightly alters the synchrony pattern (*1*). The work reported herein shows that a high degree of estrous synchrony can be attained by exposing grouped female mice to male urine for 2 days prior to pairing.

A total of 350 virgin female C57BL/6J mice, 70 to 85 days of age, were used in this experiment. The animals were weaned at 21 to 28 days of age, and placed in groups of 4 to 6 in steel cages (15 by 30 by 15 cm) until 40 to 50 days of age. Between 3 and 5 weeks prior to the experiments, new groups of 10 per cage (30 by 46 by 15 cm) were established.

Females were delegated to one of three experimental groups. Group 1 consisted of 140 females exposed to male urine for 2 days prior to pairing; group 2, 100 females similarly exposed to female urine; and group 3, 110 females constituting a control group which were neither handled nor exposed to urine. Exposure to urine consisted of placing one drop (about 0.05 ml) on the general area of the external nares 4 times daily during the 2 days prior to pairing. This treatment was given at 2-hour intervals beginning at 9 a.m. Urine was collected twice daily from mature studs in breeding colonies or from grouped virgin females of at least 70 days of age. A different set of animals provided the urine for each of the 2 days of treatment. All females from each group of ten were assigned to the same treatment.

At 9 a.m. on the day of pairing each female was placed in a clean 15 by 30 by 15-cm steel cage with one of 60 experienced stud males. On each of the following 4 mornings, females were examined for the presence of vaginal plugs. Females not showing a plug within 4 days were not used in comparing frequency distributions among the three groups.

The females in group 1 (exposed to male urine), had the highest plug frequencies on days 1 and 2, while those in group 2 (exposed to female urine) and in the control group 3 were highest on day 3 (Fig. 1). The difference between groups 1 and 2 was tested by the chi-square method and was significant ($p < .001$). There was also a significant difference between groups 1 and 3 ($p < .02$). There was not a significant difference between group 2 and group 3 ($p < .10$), although there was a trend toward delayed estrus in group 2. Seventeen (12 percent) of the females in group 1 did not show a plug within 4 days, 15 (15 percent) in group 2, and 22 (20 percent) in group 3. These differences were not significant.

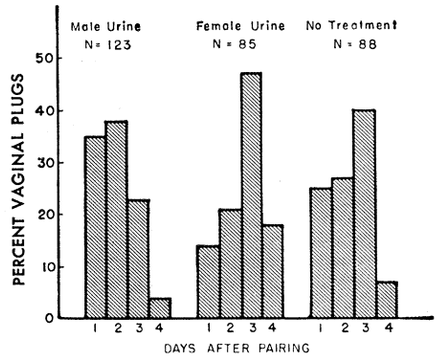

Fig. 1. Frequency distributions of vaginal plugs by days after pairing following 2 days of exposure prior to pairing, to male urine, female urine, or nothing. Values are expressed as percentages of total vaginal plugs occurring within 4 days of pairing. Sample sizes are indicated above each histogram.

It can be concluded that exposure of grouped female mice to male urine can significantly alter the pattern of estrous synchrony obtained after individual pairing with a male. A significant proportion of mice so treated achieve estrus 1 or 2 days earlier than do mice treated with female urine or untreated controls.

The role of olfaction in mammalian reproduction was reviewed by Parkes and Bruce (*2*). Pregnancy block, spontaneous pseudopregnancy, and estrous synchrony were discussed as related phenomena in that all may be the result of social-olfactory stimulation. A specific source of the stimuli has not been reported for any of these phenomena. In estrous synchrony it appears that male urine is a definite stimulus source. It remains to be determined exactly what component of urine is acting as a pheromone in the induction of estrus.

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