Using cell free conditioned medium from cultures of individual mating type strains of *D. giganteum*, Lewis and O'Day[6] showed that each strain produced pheromonal activity that could induce all of the other strains to form macrocysts. This induction of artificial homothallism was strain specific such that some strains were very strong secreters of macrocyst-inducing activity while others were strong responders. The hierarchical arrangement of inducers is: WS589 > WS606 > WS607 > WS588. This corresponds to the hierarchy of gamete formation described above. The hierarchy for responders is the exact opposite: WS588 > WS607 > WS606 > WS589.

With the recent discovery of the presumptive gamete phase of sexual development in heterothallic slime mold species and of the present relationship established between their formation and pheromone production in *D. giganteum*, future work should establish whether the gametes are the source of macrocyst-inducing factors or whether their presence reflects some other genetic component of pheromone production. Continued, critical analysis of pheromone production in other slime mold species and strains and by isolated populations of gametes should divulge the true relationship between macrocyst development, sex pheromone production and gamete formation.

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0014-4754/87/060619-03$1.50 + 0.20/0

Effects of the light-dark cycle and scheduled feeding on behavioral and reproductive rhythms of the cyprinodont fish, Medaka, *Oryzias latipes*[1]

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Summary. Medaka were maintained on a 16:8 light-dark cycle and fed once daily on one of 5 different feeding schedules. The daily rhythm of agonistic behavior rapidly entrained to the scheduled feeding time and maintained this entrainment during a 3-day starvation period. In contrast the daily rhythms of egg laying and courtship stayed entrained to the L:D cycle regardless of the feeding schedule. Thus, temporal integration of this fish with its daily environment can involve multistimuli which concurrently and differentially entrain externally expressed circadian systems.
Key words. Circadian rhythms; zeitgebers; *Oryzias latipes;* meal-feeding; behavior; environmental factors.

Behavior acts as a link between an organism's internal physiology and external environment; specific variations in physiological function produce definable and predictable behaviors. The temporal synchronization of these functions and behaviors with daily and seasonal changes in the environment is important to the individual's survival and perpetuation of the species.

The light-dark cycle has long been considered the most important environmental stimuli synchronizing or entraining circadian rhythms in plants and animals[3]. In vertebrates, including fishes, the light-dark cycle can entrain a host of endogenous rhythms including circulating hormones, locomotor activity, and reproduction[3-7]. More recently the daily eat-fast cycle has been demonstrated to be a potent synchronizer of circadian rhythms, in some cases a more potent synchronizer than the light-dark cycle. Moore-Ede et al.[4] detail research on mammals that indicate daily eat-fast cycles are more important than the light-dark cycle in entraining a number of daily patterns. Circulating glucocorticoids and locomotor activity rhythms entrained rapidly to feeding schedules but eventually all rhythms examined entrained to the feeding regime in preference to the light-dark cycle. With fishes, in contrast, although circulating cortisol and locomotor activity rapidly entrain to the daily feeding time, the rhythm of circulating thyroxine appears to remain entrained to the light-dark cycle[7].

The fact that rhythms of different physiological variables entrain to different stimuli or entrain differently (phase shift at different rates) to the same stimulus has been used as one line of evidence to support the concept of a multioscillator system of time keeping (rather than a single biological clock)[4]. It has been suggested that a multioscillator system may initially have evolved in order to provide an endogenous timing system that could simultaneously allow for rapid acclimation to short-term circadian changes in the environment (e.g., temporal change in a food resource) as well as fixed long-term circannual changes (e.g., reproduction)[7]. Presumably, reproductive functions and behaviors would be connected to a different oscillator than behavior associated with day-to-day maintenance functions. We selected an oviparous, freshwater cyprinodont, the Medaka (*Oryzias latipes*), to examine this possibility.

Methods. A population of 6 female and 4 male Medaka were placed into each of 10 35-l glass aquaria. External sexual dimorphism allowed rapid sexing both during experimental set-up and behavioral observation[8]. To avoid the continuous bottom feeding and coprophagy observed during a pilot study a plastic grating covered by $\frac{3}{8}$-inch stretchable nylon fish netting was placed on the tank bottom. This false bottom allowed feces and uneaten food to settle to the bottom of the aquaria and prevented continuous grazing and possible associated behavioral responses.

The top of each aquarium was covered by a light hood with a 25 W incandescent bulb and an automatic feeding device (details of the feeder to be published elsewhere). The light-dark cycle and feeding times were controlled by separate timers. Aquaria continuously received dechlorinated tap water (8 l/h). During a 3-week acclimation period fish were fed at random times and

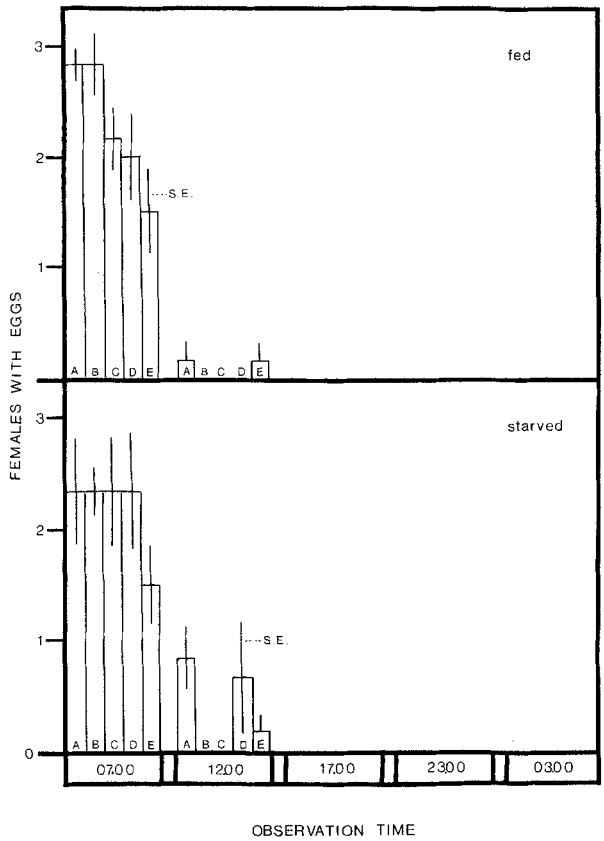

Figure 1. Daily mean ovipositioning of eggs by Medaka receiving one of 5 different feeding regimes. Fish on treatment A were fed daily at 07.00 h, B at 12.00, C at 17.00, D at 23.00 and E at 03.00. Upper graph depicts the means and standard errors (SE) of females observed at 5 1-h observation periods during the day while the animals were receiving food; the lower graph depicts the same information during a subsequent period of starvation. No eggs were noted during 17.00 h, 23.00 or 03.00 observation periods.

subjected to daily increasing light periods from 12L:12D to 16L:8D. Observations and the scheduled feeding regimes began once the final photoperiod was reached. Approximately 50 mg of Tetra Standard Mix fish food, an amount that could be totally consumed within 5 min, was delivered daily at one of 5 different times-of-day. The feeding treatment was done in replicate, 2 aquaria per feeding regime. Fish were fed either at light onset (regime A) 5 h after onset (B), 10 h after onset (C), 16 h after onset (light offset, D) or 20 h after onset (E).

Data were collected over 7 days at 17–19 °C, 3 days at 22–24 °C and 3 days of food deprivation at 22–24 °C during a 22-day period. Daily observation times were at 0, 5 and 10 h after light onset. During feeding and food deprivation regimes one set of observations was also taken at 16 and 20 h after light onset under low light conditions. Data were collected for four 5-min intervals from groups of 2 or 4 aquaria viewed sequentially during the 1-h observation period. This optimized rapid analysis of meal time effects and extended observation time per aquarium.

Behaviors recorded are listed and described in the table. Observations did not concentrate on female initiated behaviors (other than angle swimming) but these were noted when observed. Sneaky male behavior, where a 2nd male attempts to supplant the first male during courtship display, was recorded. However, occurrences were rare and were not included in the statistical analysis. Likewise male-male chase behavior was recorded as occurring near, or not near, a female. This proved to be a difficult sub-division to record reliably and thus all male-male chase behavior was combined for statistical analysis.

Description of observed behaviors

Behavior	Description
Nip-chase by male a) at female b) at male	A quick, forward thrust toward another fish followed by a quick stop once attacked fish fled. Often a 'nip' at caudal fin observed.
Pectoral fin display	2 males circle each other with pectoral fins raised.
Ovipositing	Number of females with newly deposited eggs during specified time period.
Courtship behavior	Behavior categorized by type and duration. Generally intensity ranges from category (a) to (e) but are not sequential. a) Facing: male and female b) Male approaches angle swimming female c) Circling: male swims around female d) Male behind/below female e) Peduncles touch: facing in same direction, male and female angle bodies slightly upward and touch at base of caudal fin, sometimes with quivering motion.

For the first 7 days of observation, water temperature was held at 18 ±1 °C. For the remaining days, however, water temperature was held at 23 ±1 °C (optimum for reproduction in Medaka is 21–26 °C)[8]. Although the daily patterns were not altered (comparison of means, Duncan's MRT, $p < 0.05$), warmer water temperature significantly increased (T-test, $p < 0.05$) egg laying and reproductive but not agonistic behavior. Therefore, statistical analysis and figures are based on the data collected after the water temperature was increased.

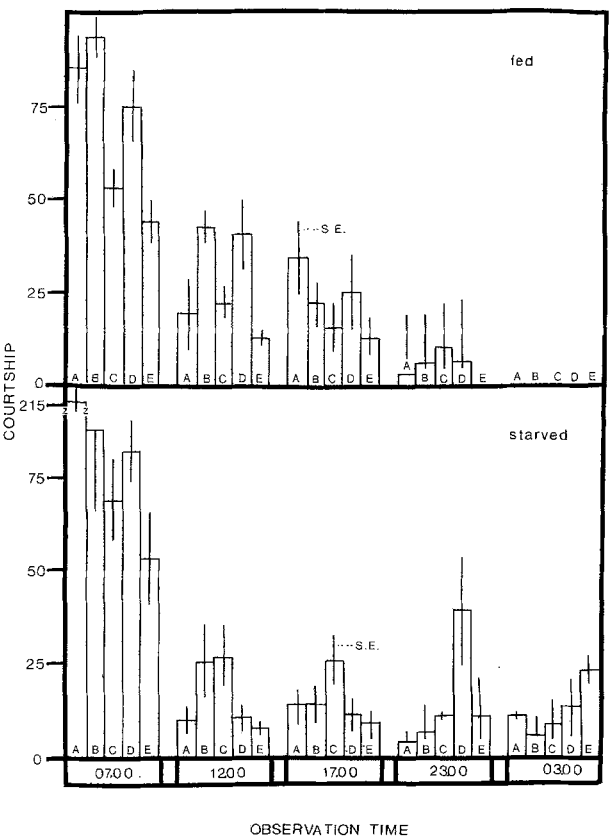

Figure 2. Daily mean courtship behavior of male Medaka receiving one of 5 different feeding regimes. Fish on treatment A were fed daily at 07.00, B at 12.00, C at 17.00, D at 23.00 and E at 03.00. Upper graph depicts the means and standard errors (SE) of a courtship intensity index recorded at 5 1-h observation periods during the day while the animals were receiving food; the lower graph depicts the same information during a subsequent period of starvation.

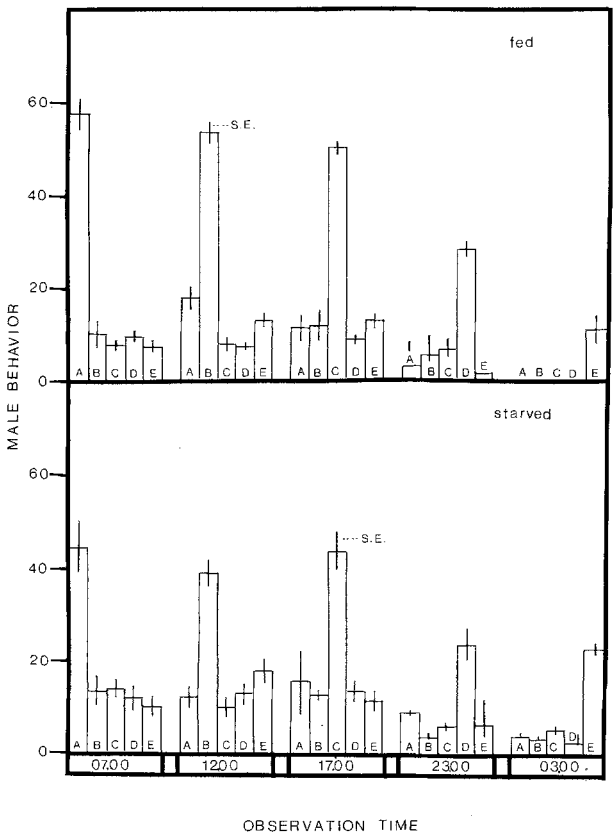

Figure 3. Daily mean agonistic behavior of male Medaka receiving one of 5 different feeding regimes. Fish on treatment A were fed daily at 07.00, B at 12.00, C at 17.00, D at 23.00 and E at 03.00. Upper graph depicts the means and standard errors (SE) of behavioral interactions observed at 5 1-h observation periods during the day while the animals were receiving food; the lower graph depicts the same information during a subsequent period of starvation.

Results. There was a significant difference (ANOVA, $p < 0.05$) between some replicates for specific behaviors. These differences could, in most cases, be ascribed to a single, highly dominant male. The total of these activities might differ between the replicate aquaria at a specific time of day or for the entire day. The variations between the replicates, however, did not affect the observed daily patterns of behavior (Duncan's MRT, $p < 0.05$). Fish on the same feeding regime displayed the same daily pattern of a specific activity. Therefore, the replicate tanks were pooled for statistical analysis.

A) Reproductive behavior and ovipositing. Fish on all but one feeding regime (C) had significant daily patterns of ovipositing and reproductive behaviors (ANOVA, $p < 0.006$) (figs 1 and 2). Peak values in all cases occurred at 07.00 h, light onset, regardless of the phase relationship of light onset to time of meal feeding (Duncan's MRT, $p < 0.05$).

The examination of reproductive behavior utilized 2 values: 1) number of females ovipositing within the observation period (as opposed to number of females with eggs still attached to anal fin (fig. 1)) and 2) courtship intensity index (C.I.I.) (fig. 2).

$$\text{C.I.I.} = \sum_{i=1}^{5} (da_i v)_i \qquad (1)$$

where i = activity type; d = duration of activity (seconds), 0–5 = 1.0, 6–10 = 3.0, 11+ = 5.0; a = number of activities of type i and duration v; v = value of activity i, facing = 0.5, female angle swims, male approaches = 1.0, male circling female = 3.0, male behind/below female = 5.0, male/female peduncles touch = 7.0.

Figure 2 displays C.I.I. values averaged over time for both feeding and fasting regimes.

In many cases at least one female already had eggs oviposited by light onset; most females that did oviposit after light onset did so within 1 h after light onset. Differences in time of meal feeding or even the absence of feeding did not affect the rhythm of reproductive behavior or ovipositing (Duncan's MRT, $p < 0.05$).

B) Agonistic behavior. Male fish on all feeding regimes also had significant daily patterns of agonistic behaviors (ANOVA, $p < 0.01$) (fig. 3). But in contrast to reproductive indices, in all cases the peak activity was at the time of meal feeding, regardless of the phase relationship of this time to the light-dark cycle. Although not a significant contribution to overall agonistic behavior (less than 10% of male-initiated aggression), female-initiated aggression was noted most often (approximately 70%) at feeding times. For all feeding regimes there was no difference in patterns of daily variation between feeding or fasting (Duncan's MRT, $p < 0.05$).

Discussion. Medaka appear to have at least two distinct circadian systems which can be concurrently entrained by different environmental stimuli. Previous research has demonstrated that the daily ovipositioning rhythm of Medaka is entrained to the light-dark cycle; the rhythm can be phase-shifted by, and exhibits transient phases to, an altered light-dark cycle[5,8]. Also the seasonal reproductive cycle in this species is accomplished through the interaction of an endogenous circadian photosensitive phase and the light-dark cycle[9]. With the exception of noting (no data) that feeding before light onset can produce a slightly earlier ovipositioning[8] these studies did not examine the possible influence of timed meal feeding on reproductive rhythms, either in phase shifting the ovipositioning rhythm, the courtship rhythm, or the photosensitive phase. Our results concur with the previous work on ovipositioning and courtship rhythms in Medaka, the peak of both rhythms occur about light onset, and, further, indicate that these rhythms are extremely resistant to the phase shifting effects of meal feeding.

Agonistic behaviors of male fish also exhibited a daily rhythm. On a random feeding regime agonistic behaviors form a broadly scattered pattern throughout the light portion of the light-dark cycle (authors, unpublished). These rhythms were, however, readily entrained to the time of food availability. This was not a response directly elicited by the presence of food as the circadian cycle remained entrained to the time of feeding for a 3-day period of starvation. Our data are in agreement with previous reports on feeding entrainment of activity rhythms in other fishes[7,10,11]. In goldfish the locomotor rhythms will remain entrained to the scheduled feeding time for a least 10 days of starvation.

It has previously been shown that daily endocrine integration within an organism can involve multiple entrainment stimuli[7]. Likewise, it is well established that the annual cycle in fishes is affected by different environmental stimuli (i.e., temperature and photoperiod)[12]. We believe, however, that this is the first study which demonstrates that temporal integration of a fish with its daily environment may involve multistimuli which concurrently and differentially entrain externally expressed circadian systems.

As has been previously pointed out there could be distinct ecological advantages to such a multientrainer system of temporal integration[7]. Thus, entrainment of resource related agonistic or locomotor behavior to the time of food availability would effect an advantage in energy conservation over indiscriminate expenditure of these energies throughout the day. With a multientrainer system, however, not all circadian systems would necessarily phase shift. In contrast, with a single entrainer system, entrainment of all systems to a single environmental stimulus,

such as feeding, might put some circadian aspects (e.g., reproduction) in a non-optimum phase with the environment.

Although a multioscillator/multientrainer system of temporal integration remains the simplest and most straight forward explanation of our results we again caution that other explanations, not addressed by the experimental design of this study, are possible[7,13,14]. For example feeding entrained rhythms could be produced by malleable (food-influenced) intervals between a single oscillator and the rhythms it controls. Or, feeding entrained rhythms may be learning phenomena dependant on a light-dark entrained time-keeping oscillator[13,14]. Studies are presently underway in our laboratory to examine this latter hypothesis.

Finally, researchers working with locomotor rhythms should take into account that the general locomotor pattern is composed of different activities (e.g. agonistic and courtship behaviors) and that these activities may be entrained to different stimuli. Ignoring this fact in experimental studies may confound interpretation of results due to noise introduced by differential phase shifts of the different activity components.

1 We thank Mark Goodrich and Steve Huber for fish maintenance and technical assistance; and Don Dovala for aid in developing the automatic feeder. The research was funded in part by NIH, AM 25191 and NIEHS, ES No.01985.

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0014-4754/87/060621-04$1.50 + 0.20/0

2,3-Dihydrolinderazulene, a new bioactive azulene pigment from the gorgonian *Acalycigorgia* sp.

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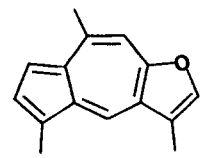
Summary. A new azulene pigment, 2,3-dihydrolinderazulene, has been isolated along with guaiazulene and linderazulene as bioactive metabolites from the gorgonian *Acalycigorgia* sp.

Key words. Gorgonian; *Acalycigorgia* sp.; guaiazulene pigments; antitumor; antifungal.

Gorgonians are rich sources of azulene pigments. Occurrence of linderazulene (**1**) was first reported by Imre et al.[2] from the Marmara Sea gorgonian *Paramuricea chamaeleon*. Fusetani et al.[3] isolated guaiazulene (**2**) from the gorgonian *Euplexaura erecta* collected at Enoshima, Japan. Subsequently, Scheuer's group[4] discovered a number of halogen- and nitrogen-containing guaiazulenes in addition to **1** and **2** from a deep sea gorgonian of Hawaiian waters. In our study of biologically active substances from marine organisms occurring in Okinawan waters, an extract of the gorgonian *Acalycigorgia* sp. showed antitumor activity against P388 mouse leukemia cells. Separation of the extract gave three guaiazulene pigments (**1–3**) as active constituents. A sample (600 g) of *Acalycigorgia* sp., collected at Cape Zampa, Okinawa, in April, 1985, was extracted by steeping in acetone. The acetone extract was concentrated, and the resulting aqueous suspension was extracted with ethyl acetate to give 3.4 g of an oil. A part (2.7 g) of the oil was chromatographed on silica gel with heptane-ethyl acetate (10:1) to furnish two portions. The first portion (1.02 g), containing blue pigments, was placed on a bed of reverse phase adsorbent (RP-8) in a sintered glass filter and successively eluted with methanol and acetone. The same filtration was repeated with the methanol eluate to give 320 mg of a mixture containing the pigments. The mixture was then separated on a Lobar Si-60 column with heptane-ethyl acetate (19:1) into 5 fractions. Each of the 3 pigment-containing fractions was further separated by preparative TLC on silica gel (heptane-ethyl acetate 30:1 to 10:1) to give 3 pigments. Purification of these pigments by HPLC (Hibar Si-60, heptane-ethyl acetate 40:1 to 20:1) furnished 93 mg of guaiazulene (**2**) as a blue oil, 7.8 mg of linderazulene (**1**) as purple crystals, m.p. 105.5 °C (lit.[5] m.p. 106–107 °C), and 47 mg of the new compound **3** as a purple oil, $[\alpha]_D$ + 800° (c 0.05, $CHCl_3$).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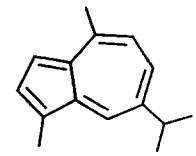
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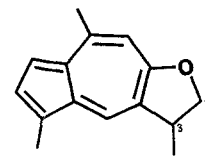
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Compounds **1** and **2** were identified as linderazulene and guaiazulene, respectively, by comparison of spectral data with those reported for these pigments[2,3]. The molecular formula, $C_{15}H_{16}O$, of the new, optically active pigment (**3**) was deduced from high resolution EIMS[6] at m/z 212.1202 (Δ 0.1 nm). The ^{1}H NMR signals for the azulene portion [acetone-d_6, δ 8.10 (1 H, s), 7.26 (1 H, d, J = 3.8 Hz), 7.15 (1 H, d, J = 3.8 Hz), 6.69 (1 H, s), 2.73 (3 H, s) 2.57 (3 H, s)] were similar to those of **1**. The remaining resonances, a methyl doublet at δ 1.40 (J = 6.8 H), a methine multiplet at δ 3.72, and methylene double doublets at δ 4.69 (J = 8.8, 8.8 Hz) and 4.14 (J = 8.8, 6.8 Hz), suggested that **3** was 2,3-dihydro-derivative of **1**. The ^{13}C NMR data[6] [δ 77.93 (t, C-2), 39.96 (d, C-3)] also supported this conclusion. Structural confirmation was provided by dehydrogenation of **3** to **1**, as follows. A