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Shedding by rod photoreceptors after sunrise in fish

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Abstract. Diurnal shedding by retinal rods was studied in wild cutthroat trout, *Oncorhyncus clarki*, hatchery rainbow trout, *Oncorhyncus mykiss*, and the plains killifish, *Fundulus zebrinus*, by counting the shed tips of rod outer segments ingested as 'phagosomes' by pigment epithelial cells. After sunrise, phagosomes increased in all species, but fewer occurred in trout, and these were elevated from 3 to 9 hours after sunrise. Shedding occurred earlier in the light period and was more robust in killifish, with phagosomes elevated from 1.5 to 6 hours after sunset. The data suggest that both production of phagosomes by shedding and their subsequent disposal are slower at the lower temperatures experienced by trout. Otherwise, rod shedding produced under natural lighting is not appreciably different than that provoked by sudden onset of artificial light.
Key words. Trout; killifish; rod photoreceptor shedding; sunrise.

Vertebrate retinal rod outer segments (ROS) are continually renewed by addition of new discs which replace those shed from their tips. The shed tips are injested by retinal pigment epithelial cells (RPE), where they appear as phagosomes until they are degraded[1]. Generally, a burst of phagosomes originating from rods occurs within 1–6 hours after light onset following darkness, while a lower level of spontaneous shedding occurs at other times in the diurnal cycle. This diurnal pattern of rod shedding has been identified in goldfish[2], skate[3], brown trout[4], leopard frog[5], clawed toad[6], newt[7], chicken[8], rat[9], and the mouse[10]. For some species, the diurnal pattern of rod shedding persists in continual darkness, indicating a circadian component which may be reset by light onset, while in others, rod shedding appears responsive only to light onset[11].

Cone photoreceptors generally shed their tips synchronously after light offset[12], so that phagosomes originating from cones are present during the dark period and temporally isolated from those originating from rods. In diurnal species such as goldfish[2], chicken[8] and tree squirrel[13], there are two prominant peaks of shedding, after light onset for rods, and after light offset by cones. There are exceptions, notably the cat, in which both rod and cone shedding is triggered by light-onset[14], and the tree shrew[15], in which cones shed at light onset. Since rod shedding in thought to maintain a functional rod outer segment volume, the need to understand its relationship to cyclic lighting has encouraged investigation. However, most studies have utilized sudden or 'rectangular' transition between total darkness and various levels of artificial light in the laboratory to produce shedding episodes. Few have attempted to provide gradual light transitions that permit nocturnal species to avoid strong light, or which allow photomechanical repositioning of rods, cones and RPE melanosomes of lower vertebrates to be completed during, not after light change. In the latter, sudden light onset would immediately expose rods to stronger than normal bleaching of visual pigment[16, 17]. Furthermore, rectangular light-dark cycles may reduce or even eliminate seasonal or circadian cues associated with dawn and dusk periods[18]. Therefore, it is conceivable that the use of rectangular lighting might induce shedding responses which are unrepresentative of responses provoked by natural cyclic light.

Relatively few studies have attempted to define rod shedding in natural light, and for various reasons conclusions remain tentative. In the skate, *Raja*, which has only rods and is assumed to be a deep water nocturnal genus, a small (2×) increase in phagosomes occurred after sunrise in individuals sampled from a shallow outdoor pen which may have allowed them cover from downwelling spacelight[3]. In the same study, a 12L/12D rectangular light protocol produced more abrupt light-onset shedding. Recently, Ripps and Dowling[19] concluded that some smaller rods function in bright light, and thus, the skate may not be limited to scotopic behavior as originally thought. McCormack et al.[20], in a review of earlier work[4], reported robust shedding peaks over two days in brown trout in 'natural' light, but the peaks did not coincide at the same time each day after light-onset, peaking one day at late afternoon (L10-L14) and the next day immediately after light onset (L0–L4). The reported light intensity (30 Lux), indicates that the fish were held indoors. Bassi and Powers[21] recorded light-onset shedding by rods in goldfish kept in an indoor aquarium illuminated only by a laboratory window and in 12L/12D rectangular protocol. The shedding they report in both cases was more moderate

than that reported in two earlier studies on goldfish utilizing rectangular light[2,22], but shedding appeared to be more dramatic under rectangular light. Finally, Dahl and Gordon[23] found that a moonless light followed by sunrise produced increased phagosomes one hour after sunrise in frogs previously entrained to 14L/10D rectangular light, but their experimental design lacked the repeated sampling necessary to define a diurnal shedding pattern.

Through much has been learned from nearly two decades of study of the pattern of rod photoreceptor shedding in relation to cyclic light, the question of whether rectangular light onset represents a 'supranormal' stimulus to shedding was raised but not resolved. However, data gained through the use of rectangular light protocols would be beyond reproach if significant rod shedding could be demonstrated in natural light. With this in mind, we examined phagosome production after sunrise in cutthroat trout, *Oncorhyncus clarki*, rainbow trout, *Oncorhyncus mykiss*, and plains killifish, *Fundulus zebrinus*.

Methods

Cutthroat trout were sampled from Camp Creek, a tributary to the North Santiam River, Linn County, Oregon on August 15–16 (Fork length: 14.5 cm ± 1.8 (SEM), latitude 44° 30′, elev. 914 m, first light at 05.30 h, sunrise 06.30 h, daylength 13 h 20 min, water temp. 14.5 °C) and from Lookout Creek, a tributary to Blue River, Lane County, Oregon on August 27–28, (Fork length 11.7 cm ± 0.97, latitude 44° 15′, elev. 609 m, first light at 05.45 h, sunrise 06.45 h, daylength 13 h, water temp. 14.5 °C). Both of these streams are small, clear, and are canopied by old growth Douglas fir, with alder and vine maple understory. During sampling, skies were clear in both locations, with less than 10% skylight available. Moonlight was minimal in both locations. Fish were caught by barbless fly angling (#14 McKenzie Special) and retained in a 3 cubic foot wire mesh trap in the stream for subsequent sampling.

Rainbow trout were netted periodically from a hatchery raceway in the Kamas State Hatchery, Summit County, Utah, July 12–13 (Fork length 9.75 cm ± 0.47, latitude 40° 40′, elev. 2130 m, first light 05.00 h, sunrise 06.00 h, daylength 14 h 30 min., water temp. 11 °C). The raceway was in a large clearing with security lighting turned off for 10 days prior to sampling.

Killifish were captured with a common sense seine and placed in a stream cage for sampling on June 20–21 from the Pecos River, a brackish, clear stream in Crane County, Texas (Fork length 4.62 cm ± 0.69, latitude 31° 18′, elev 832 m, first light at 05.00 h, sunrise at 06.00 h, daylength 14 h, water temp. 26 °C).

Light intensity was tracked with a portable light meter having a dynamic range of 6 log units from full sunlight to partial moonlight. Civil twilight (the time between sunup or sundown and the sun's position at 6° below the horizon) was 37 min in all locations, and first light occurred at 4.5 log units down from full daylight streamside in forest locations, and 5.4 log units down from full daylight at the Kamas hatchery and the Pecos River. In the latter two locations, a full moon was present during the night at 5.4 log units down from full daylight until 02.00 h. For clarity, we use the convention 12L/12D to denote light and dark portions of a 24 diurnal cycle, and denote specific times during diurnal cycles by L or D, followed by the hour or duration in hours (e.g., L4, or L1–L4).

For the trouts, 3–4 fish were sampled at various times during the following night and day. For killifish, 1 fish was taken at each sample time. Eyes were removed on site in ambient light and immediately placed in a cold, 87 mM phosphate buffered (pH 7.2) mixture of 2% glutaraldehyde 2% paraformaldehyde with 3% sucrose added. A slit was made in each eye to assist penetration of fixative. During the night, capture and enucleation were assisted by use of a flashlight fitted with a deep red filter (Wratten Series 1). After a few hours, the anterior segments were removed and the posterior segments pared to leave 1 mm of tissue on either side of the optic nerve entry. Eye segments were washed in buffer, post-fixed for 1 h in 1% osmium tetroxide, washed and dehydrated through a series of ethanols to propylene oxide. Material was embedded in a mixture of Epon/Araldyte resin and blocks were faced parallel to the long axes of ROS. Sections were made at 1 micron on an MT-2 ultramicrotome and transferred to a drop of freshly filtered 0.1% toluidine blue dye in 0.5% Sodium Borate, diluted to achieve optimal staining when dried down (1–2 min) on a 105 °C hot plate.

For each eye, a retinal section was examined at six or more locations at 1000 x, and phagosomes counted over a 100 um field, using a Leitz Laborlux S equipped with an ocular micrometer and MPS 46 camera. The average of the values among all locations analyzed served as an estimate for the eye. In killifish, both eyes were used and the average of two eyes reported for each sample time. In trout, the data for one retina from 3–4 fish were averaged for each sample time. Photomechanical positions of rods, cones and melanosomes were noted and the pigment index was reported to track light adaptation[16].

Results

The pattern of shedding would appear to be a more reliable basis for comparison among taxa than total numbers of phagosomes, given that the density and/or volume of ROS varies among species. Amphibian ROS are 2–3 times larger in diameter than most teleost ROS[24,25]; thus fewer, larger phagosomes could account

for the same volume of material shed by a given number of teleost rods. Trout rods are about 4/5 as dense as kilifish rods, but the same diameter (3 μm) and a slightly greater number of trout rods per 100 μm would shed to match numbers of phagosomes originating from a comparable area of killifish retina. This scaling problem would be obviated by reporting phagosomes per 100 ROS[8], but most previous investigators have preferred to use a spatial density (phagosomes per 100 μm). In some cases, a census of different sizes of phagosomes has been reported, to differentiate those originating from light-onset from smaller phagosomes thought to comprise spontaneous or 'background' shedding[26]. Similarly, phagosomes have been graded according to their life history; i.e., reported as 'freshly shed' according to such criteria as shape, size, proximity to rod tips and/or density of stain[27]. In this study, we report all phagosomes (>1 μm) per 100 μm width of RPE, to facilitate comparison with the previous reports. In both trouts and killifish there was an increase in the number of phagosomes after sunrise (figs 1, 2). Some phagosomes could be observed breaking from the ROS tips, and previous studies have demonstrated that most phagosomes observed after light onset originate from ROS[11]. At first glance, the shedding of ROS after sunrise in trout appears to be relatively modest in comparison to that observed in killifish (to a peak of 3.1 and 4.2/100 μm in cutthroats, to 7.2/100 μm for rainbows, and to 21.2/100 μm in killifish, fig. 2.). However, the increases over phagosome counts in prior dark samples were 4–6-fold in cutthroats, 7-fold in rainbows and 5-fold in killifish (fig. 2). The ratios are similar to those seen in other studies, including those which have separated phagosomes originating from cones[2,5,22].

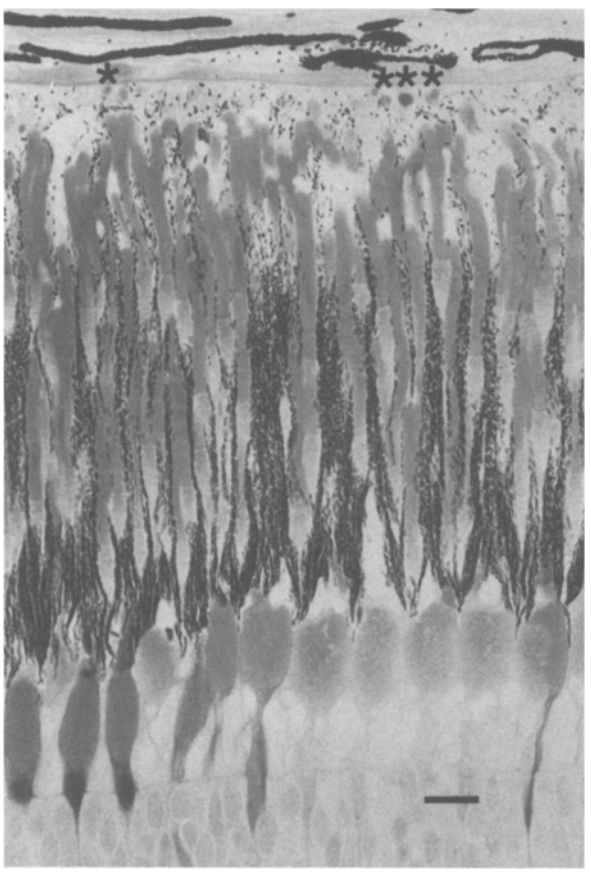

Figure 1. Phagosomes [below *] appear as small, darkly stained bodies at the schlerad border of the RPE, near the tips of ROS, 4 hours post-sunset in the rainbow trout, *Oncorhyncus mykiss*. Scale bar, 10 μm.

The relatively low numbers of phagosomes overall in trout as compared to killifish may be due to low temperatures experienced by trouts (14 and 11 °C vs 26 °C). Low temperature not only could delay shedding by slowing synthesis of new ROS discs[11], but could also delay disposal and clearance of phagosomes from the pigment epithelium. The latter possibility is pointed to by the fact that phagosomes increased more slowly and remained elevated longer in trout (from L3 to L9, fig. 2, table). The comparison is valid because neither the trout nor killifish were near the upper limit of their thermal tolerance.

A review of studies which have included sampling points sufficient to define shedding of ROS after light onset supports the view that both shedding and disposal of phagosomes are slowed by low temperatures. Maximum phagosome numbers are usually higher (26/100 μm), peak within 1–2 hours of light onset (L1–L2) and disappear from the RPE more quickly (by L4) in the rat[9,31,32], opossum[27], cat[14] and chicken[8], all animals with body temperatures in the 37 °C range and high metabolic rates. Given that ROS renewal, shedding and RPE disposal are supported by metabolic processes, one would expect fewer phagosoms to arise after light onset and remain visible in the RPE over a longer period of time in lower vertebrates held at lower ambient temperatures.

Conversely, at higher temperatures, shedding in these animals should approximate the pattern seen in mammals. This supposition is supported by a review of previous studies on lower vertebrates (table). In particular, the work of Hollyfield et al.[26] showed a direct correlation of phagosome number with temperature in tadpoles of the leopard frog, wherein peak numbers at 23, 28 and 33 °C were 19, 35 and 42/100 μm, respectively, and phagosomes remained elevated only until L4. A similar pattern was shown by the clawed toad at 23 °C (31/100 μm; table). Phagosomes were also high in goldfish at 25 °C (25–30/100 μm at L1–L2) and in killifish at 26 °C (21.2/100 μm at L1–L2), but in killifish they remained elevated longer (to L6, table). Fewer phagosomes were produced later and disappeared more slowly in trout sampled from 14.5 and 11 °C (with peaks from 3-7/100 μm at L3.25 to L5.5, remaining elevated until L9, table, fig. 2). Finally, in the skate, phagosomes increased to 3.8 and 4.4/100 μm after gradual and sudden onset respectively

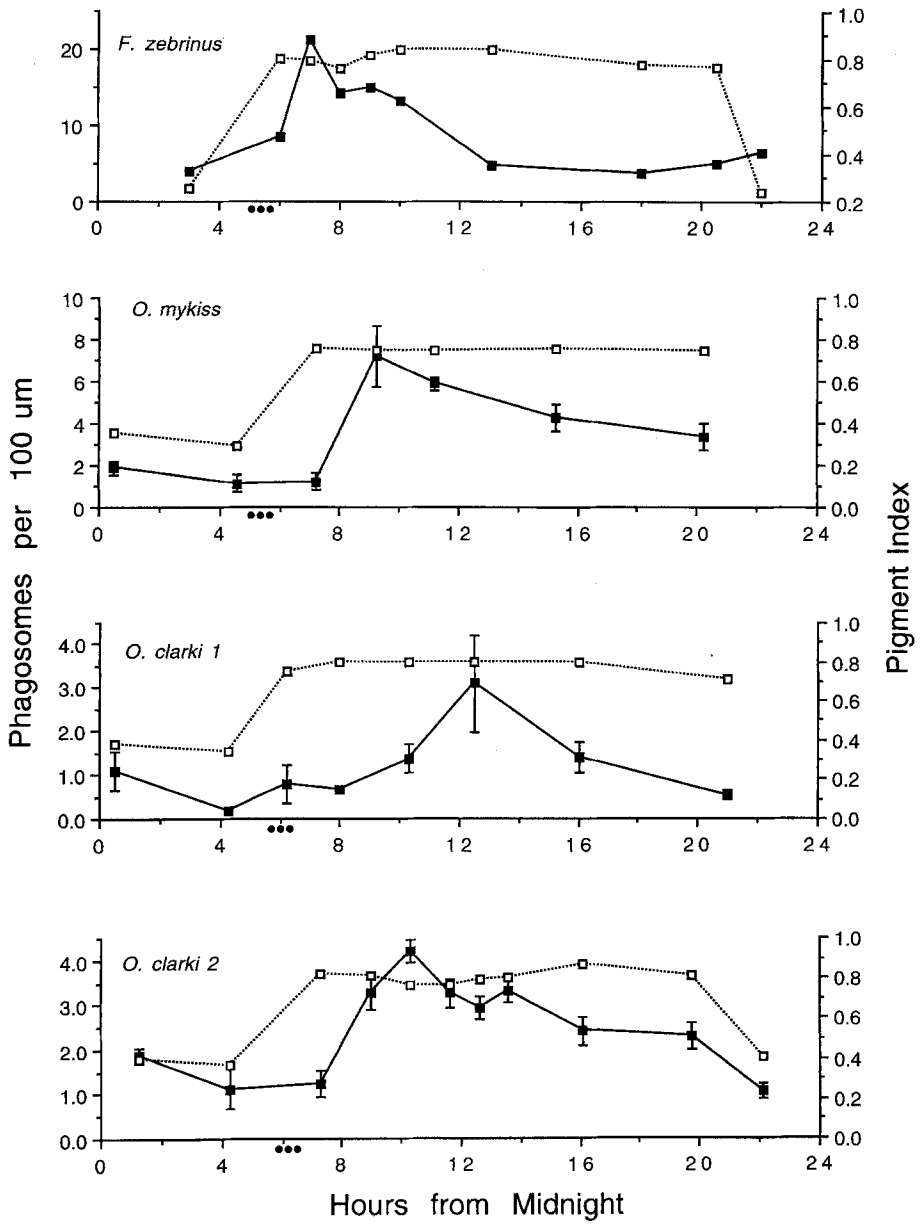

Figure 2. Number of phagosomes [■] and pigment index [□] in the retinae of *F. zebrinus*, *O. clarki* (*1* Camp Creek, *2* Lookout Creek) and *O. mykiss*, sampled during a diurnal period. SEM of phagosomes counts shown for *O. clarki* and *O. mykiss* (3–4 fish per point). *F. zebrinus* samples are the mean of both eyes of one fish. [· · ·] indicates first 45 minutes from first light.

in cold sea water, and were elevated from L0–L6 (table).

Some data is inconsistent in supporting the effects of thermal history proposed above. It is not clear why the peak phagosome numbers reported by Basinger et al.[5] for the frog are lower at 23 °C (8/100 µm at L1) and remained elevated only from L0.5–L1.5, or why in one goldfish study (8.5/100 µm)[21], peak phagosome numbers do not reach values expected at a room temperature (22 ± 2 °C; Bassi, pers. commun.). Even more troubling, data reported for brown trout shows that large phagosomes reached robust peaks at two different times of day in both adult (L10–L14 and L2.5–L6) and juvenile trout (L0 and D3–D6)[20], though these fish may have been kept near their limit of thermal tolerance (photomicrographs of phagosomes and temperature information are not reported). These exceptional results reinforce the view that shedding can be triggered by events other than light onset.

Shedding could be delayed during gradual light onset or natural sunrise if there is a threshold for shedding above that of first light, but this idea is not supported by present data. Shedding appeared to be delayed in cutthroat trout (peaks at L5.5 and L3.5) and in rainbow trout (peak at L3.25), well beyond sunrise and photomechanical light adaptation (fig. 2). However, phagosomes were elevated within 1–2 hours of sunrise in killifish (fig. 2), goldfish and skates (table). One can therefore

A review of shedding by retinal rods in lower vertebrates, as studied by serial sampling during light (L) and dark periods (D) and under rectangular (L/D) or gradual light onset. Phagosome counts converted to a 100 µm field. Data not reported or defined (– – –).

Species & daylength	Phagosomes/100 µm min/max/duration	No./Sample	Samples/24 h	Temp. (°C)
Goldfish[a]	0/25@L1	4	24	25
12L/12D	L0.5–L5			
Goldfish[b]	1/25–30@L4	1	20	25
12L/12D	L1–L6			
Goldfish[c]	0.2/8.5@L1	1–2	9	– – –
12L/12D	L1–L6			
lab window only[d]	0.1/3.5@L2	1–2	10	– – –
	– – –			
Brown trout[e]	0/22@L10–L14	10	13	– – –
	L10–L14			
(1 yr old)	0/20@L2.5–L6			
	– – –			
(9.5 week old)	1.5/32@LO	10	13	– – –
	L0–L4			
	12–20@D3–D6			
Cutthroat trout*	0.5/3.1@L5.5	3–4	8	14.5
	L3–L9			
	1.0/4.2@L3.5	2–3	11	14.5
	L3–L9			
Rainbow trout*	1.1/7.2@L3.25	4	7	11.1
	L3–L9			
Killifish	3.8/21.2@L1.5	1	10	26.0
	L1.5–L6			
Skate[f]	2.2/3.8@L7	3–4	14	cold
outdoor	L0.5–L6.5			SW
12L/12D[g]	0.5/4.4@L1–L3	3–4	15	cold
	L0–L6			SW
Frog[h]	0/8@L1	1	9	23
14L/10D	L0.5–L1.5			
Frog[i]	7.5/19@L2	3	11	23
12L/12D	L1–L4			
Tadpoles	1/35@L4	3	11	28
	L1–L4			
	5/42@L2	3	11	33
	L1–L4			
Clawed toad[j]	7/31–L1	3	4(×3)	28
12L/12D	L1–L4			
Newt[k]	0.2%/0.8% RPE@L5	3	8	15
12L/12D	– – –			

I = incandescent, F = flourescent, N = natural lighting

[a] *C. Auratus*; ref. 2, fig. 2; 700 Lux F.
[b] *C. Auratus*; ref. 22, fig. 8; 32 Lux I.
[c] *C. Auratus*; ref. 21, fig 2; 320 Lux F (86 µW/cm^2).
[d] *C. Auratus*; ref. 21, fig. 2; N (window) 12L/12D (3162 µW/cm2).
[e] *Salmo trutta*; ref. 20, fig. 2e, f; N 14L/10D@30 Lux (indoor?)
[f] *Raja*; ref. 3, fig. 10; N. (outdoor pen).
[g] *Raja*; ref. 3, Fig. 10; 85 Ft. C F.
[h] *Rana pipiens*; ref. 5, fig. 2; 60 Ft. C F.
[i] *Rana pipiens*; ref. 26, fig. 5, 6; 20 Lux I, large phagosomes only.
[j] *Xenopus laevis* larvae; ref. 30, fig. 4; 200–250 Lux I, phagosomes > 2 µm.
[k] *N. virescens*; ref. 7, fig. 8, 624 Lux F.

*Natural habitat: see fig. 2 legend.

conclude that sunrise does not necessarily force a delay in shedding, and can produce 'burst' shedding at L1-2, similar to responses observed previously under rectangular light onset (goldfish, frog, clawed toad and newt (table); the chicken[8], and mammals[9, 10, 14, 27]). The picture is complicated in certain species by circadian factors. For instance, mice have exhibited a robust peak of phagosomes shed by rods 1.5 hours following sudden light onset[10], but rod shedding can increase significantly prior to light onset (at D8 or D10)[28, 29].

The results of this study and a comparative analysis lead us to conclude the following:

1) that light-onset shedding as defined under rectangular light protocols is not 'supranormal', and

2) that the pattern of shedding as well as its magnitude are affected by the temperature of the eye.

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