

FY96 Investigational Report :

***Renibacterium salmoninarum* and *Nanophyetus*
Metacercaria Infection in Adult Chinook Salmon:
Trinity River Hatchery Broodstock (1992-94, 1996),
Klamath Estuary Net Harvest and KMZ Ocean Sport
Catch in 1996.**



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Summary: Kidney tissue from spawned, Trinity River Hatchery (TRH) broodstock (1992-94, 1996), and both ocean caught and lower Klamath R. estuary net harvest adult chinook (1996) were assayed for metacercaria parasites and Renibacterium salmoninarum (RS) antigen. The TRH adults had a high incidence of metacercarial infection ($\geq 92\%$) with the Fall-run fish having 5 - 1 OX more severe infections than Spring-run chinook. The mean severity value ranged from 204 to > 5,000 metacercaria per gram of kidney tissue. The severity of infection for both stocks dropped in 1996. The majority of metacercaria infect the adults during the up-river phase, as fish captured in the ocean or lower estuary had little or no detectable metacercarial infections. While the adult trematode has not been experimentally produced for identification, it is believed that the observed metacercaria are *Nanophyetus salmincola*. The majority of samples had only low-levels of RS antigen suggesting that Bacterial Kidney Disease (BKD) was not prevalent in the sampled populations. Fish captured in both the ocean or estuary had the lowest RS antigen profile of all of the sample groups. Neither of these fish pathogens were judged to significantly effect the survival of adult chinook in the Trinity River, however, sample biases limit the confidence of this conclusion.

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Introduction

Trinity River State Fish Hatchery (TRH) is operated by the California Department of Fish and Game with funding administered by the Bureau of Reclamation. The hatchery is located below Lewiston dam. Two runs of chinook salmon (spring and fall), coho salmon, and steelhead trout are reared at TRH and are the progeny of broodstock captured and spawned at the hatchery.

The California - Nevada Fish Health Center (FHC) surveyed Trinity River chinook salmon (*Oncorhynchus tshawytscha*) juveniles and adults for health and physiological measurements in 1991-1994 and again in 1996. Both natural and hatchery juveniles have been examined in the spring and fall. Two common and significant fish pathogens detected in both natural and hatchery out-migrants are the trematode *Nanophyetus salmincola* and the bacterium *Renibacterium salmoninarum*.

Trinity River Hatchery (TRH) chinook are exposed to the trematode parasite soon after their release into the river and can sustain heavy infections within days. Both the incidence of infection and severity tend to be higher in fish released in the fall compared with the spring. The severity of infection can range up to 33,000 metacercariae per gram of kidney in juvenile chinook. *Nanophyetus salmincola* is found in salmonids and other associated freshwater fish (cottids, cyprinidae, lamprey) throughout the Pacific Northwest (Millemann and Knapp 1970). Its range is limited to waters which are habitat for its intermediate host, the Juga sp. snail. The pathogenicity of the trematode to fish is reported to vary considerably and appears to be a function of rate of accumulation, fish size, species and stock susceptibility, infection site(s), and absolute number of metacercariae (Newcomb et al. 1991, Wood and Yasutake 1956, Baldwin et al. 1967, Milleman and Knapp 1970). Human cases of nanophyetiasis have been reported (Harrell and Deardorff 1990). The trematode is itself parasitized by the rickettsia, *Neorickettsia helminthoeca*, which is responsible for salmon poisoning disease of dogs (Schmidt and Roberts 1981)

The life cycle of *N. salmincola* starts with the release of eggs from the adult trematode into the intestine of its final host, a piscivore such as an otter, bear, raccoon, heron, merganser, etc, and pass out into the water with feces. A ciliated miracidium stage hatches from the egg, penetrates a snail host (*Oxytrema* = Juga sp.), asexually multiplies, and eventually produces a xiphidiocercaria (cercaria with oral sucker stylus which is motile by use of its tail). The cercaria will seek out a fish host and rapidly burrow into the skin, lose its tail, and migrate through the circulatory system to various tissues such as the gill, heart, liver, muscle, optic nerve, and kidney. The parasite (now referred to as metacercaria) tends to concentrate in the posterior kidney, probably due to the migration path through the renal portal system (Millemann and Knapp 1970). The metacercaria will remain with the Salmonid fish throughout its salt water phase and will complete its lifecycle when the fish is eaten by a final host. The longevity of the

metacercarial stage has been used as a biological tag for steelhead caught in the central Pacific ocean (Dalton 1991).

Renibacterium salmoninarum is the causative agent of Bacterial Kidney Disease (BKD) and is an obligate pathogen of Salmonid fishes worldwide (Evelyn 1993). This bacterium can survive and multiply in the host's phagocytic cells. Transmission can be both horizontal (fish - fish) and vertical (female - progeny). BKD is a chronic disease characterized by granulomatous lesions in the kidney and is frequently fatal. Banner et al. (1986) reported that ocean-caught salmon can have BKD. The CA-NV Fish Health Center has observed that > 70 % of both the natural and hatchery chinook smolts out-migrating from the Trinity River will have low levels of R.salmoninarum antigen in their kidneys.

The high incidence of severe metacercarial infections and low-level Renibacterium salmoninarum infection observed in Trinity River smolts prompts the question of whether these pathogens will significantly impair the health and performance of chinook smolts and decrease their survival.

Method and materials

Sample method and sites - Kidney tissue, 1 - 3 cm² in size, was removed with clean dissection tools from the mid-kidney of freshly-killed adult chinook. The tissue was placed in whirl-pak bags and frozen until processed for metacercaria counts and ELISA. The identity of each sample was recorded and included sample location, date, head tag number for ocean and estuary samples, and age (jack = small 2 year old male, or adult = 3 - 5 yr. old male or female). Kidneys collected in 1992 - 1994 were from spawned fish at TRH. In 1996, samples were from 3 sources: TRH broodstock (Mel Willis, CDFG Pathologist), net harvest monitoring in the lower Klamath estuary (Yurok Fisheries Program), and ocean sport catch in August and September. The ocean samples were collected dockside from anglers at 4 locations: 1) Trinidad, 2) Crescent City, 3) King Salmon (Eureka), and 4) Fields Landing (Eureka).

Histology - In 1993, additional kidney samples were fixed for 24 hrs. in Davidson's fixative (Humason 1979), transferred to 70 % ethanol, processed for 5 μm paraffin sections, and stained with hematoxylin and eosin. Tissue abnormalities and metacercaria were evaluated by light microscopy.

***R. salmoninarum* ELISA - Kidney tissue was diluted 4x (w/v) in PBS-0.05% tween20, homogenized, boiled for 15 min., centrifuged, and the supernatant tested for antigen. In 1994, Spring-run chinook tissues were mistakenly diluted 16x. Commercial polyclonal antisera, to whole cell preparations, was used for the assays. The optical density (O.D. = absorbance at 405 nm) of the labeled antibody- antigen reactions were averaged between sample replicates. A blank well O.D. value was subtracted from each sample. Comparisons between groups**

were made with log transformed O.D. values. Three semi-quantitative categories are used to view the transformed data: 1) BNC = sample O.D. Below the Negative kidney Control value, 2) Suspect = sample O.D. above BNC and less than -0.7 (= Log 0.2., O.D. of 0.2 is the subjective value which there is the likelihood of confirming infection by FAT), and 3) Positive = sample O.D. > -0.7 .

Metacercaria counts - In order to standardize the severity of Infection measurement, the number of metacercaria per gram of kidney examined was determined for each sample. In 1992 - 1994, the frozen kidney sample was dissected into thin slices and several slices placed onto a tared glass slide (50 x 25 cm). After the weight of the tissue was recorded to the nearest 0.001 gram, another glass slide was press down on top of the tissue and the number of metacercaria in the squash preparation counted with a binocular dissection microscope at 50 - 70X magnification . Observations on the focal distribution of the metacercaria prompted us to modify our methods to avoid over or underestimating the infection severity of a particular sample. This bias would only be significant in light infections which were rare in hatchery returns. In 1996, the entire kidney sample was homogenized within the whirlpak bag into a "slurry" and a drop of this slurry placed on the tared slide.

Statistics - Data analysis / Statistics- Group data was tested for normality and either analyzed by the parametric (T-test, I-way ANOVA) or non-parametric tests (Mann-Whitney rank sum test, Kruskal-Wallis ANOVA on ranks). If significant differences among the groups were detected in the ANOVA tests, Student-Newman-Keuls multiple comparison (pairwise) tests were performed to identify which group was different. An alpha (type I error) value of P_i , 0.05 was chosen for all tests. Both Lotus 1-2-3 "spreadsheets and SigmaStat™ software was used for data manipulation and analysis.

Results and Discussion

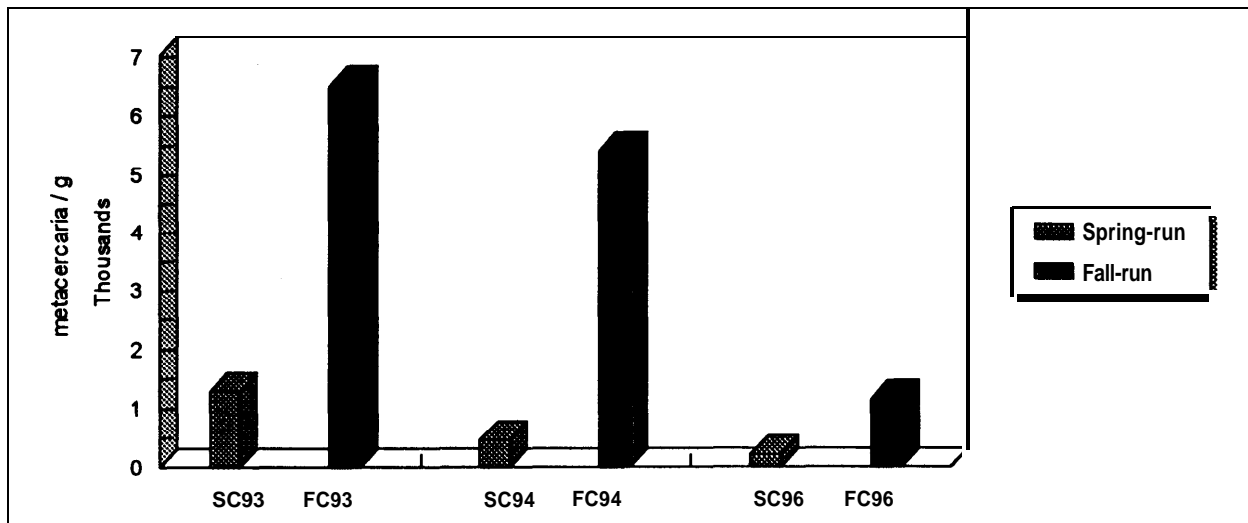
Metacercaria - The TRH broodstock had a high incidence (92 - 100%) of heavy metacercarial infection (Table 1). Fall-run adults had an average of 5 - 10X more metacercaria per gram of kidney than Spring-run (Fig. 1). The average intensity of infection ranged from a low of 204 metacercaria / g in the 1996 TRH Spring-run to over 5,000 in the 93 and 94 Fall-run. The highest intensity of infection recorded was 23,330 metacercaria /g kidney in a 1994 Fall-run adult. Grossly, many of these kidney samples had a "salt and pepper" appearance due to the large number of metacercaria cysts. This trend continued in 1996 even though the overall severity of infection dropped in both stocks when compared to 1992-94. We observed similar reductions in metacercaria infection severity in juvenile chinook collected in the spring and fall of 1996. I believe that the reduction in metacercarial infection in 1996 could be due to scouring of the intermediate host population during high winter flows. In a separate study (Foott et al. 1996). it was very difficult to collect Juga sp. snails from multiple sites in the Trinity River in 1996.

Table 1. Metacercaria infection in adult Spring-run (SCS) and Fall-run chinook salmon (FCS) from TRH broodstock (1992-94, 1996) and ocean / estuary samples collected in 1996 (96 OCEAN). In 1992 - 1994, the age of the sampled fish was tracked as 2 yr. old male (Jack) or +3 yr. old female or male (Adult). Incidence data reported as number of positive samples / total samples collected for that year (%). Severity of infection data reported as metacercaria (metac.) per gram of kidney examined.

Broodstock	Incidence of Infection (%)	Mean (SEM) Metac. / g	Median Metac. / g	Maximum Metac. / g
92 SCS	60 / 60 (100)	NA**	NA **	NA**
Adult Jack	30 / 30 (100) 30 / 30 (100)	3961 (\pm 1261) 7458 (\pm 1374)		9000 14100
93 SCS	40 / 40 (100)	1262 (+ 149)	906	3632
Adult Jack	20 / 20 (100) 20 / 20 (100)	1310 (+202) 1209 (\pm 225)	1042 832	3405 3632
93 FCS	40 / 40 (100)	\geq 5000 ++	ND	> 5000
Adult Jack	20 / 20 (100) 20 / 20 (100)			
94 SCS	56 / 61 (92)+	462 (+ 83)	195	3145
Adult Jack	11 / 14 (79) 27 / 27 (100)	363 (\pm 129) 746 (\pm 155)	176 528	1337 3145
94 FCS	57 / 61 (93)	5362 (+ 650)	3928	23330
Adult Jack	30 / 30 (100) 27 / 31 (87)	5445 (\pm 917) 5282 (\pm 936)	5395 3548	23330 16450
96 SCS	44 / 45 (98)	204 (\pm 29)	128	723
96 OCEAN	2 / 54 (4)	6 (\pm 0.2)+++	6	6
96 FCS	60 / 60 (100)	1134 (\pm 97)	1026	3422

- NA** Not Applicable as only limited metacercaria / g data collected in 1992 (9 jacks & 6 adults).
 +++ Statistics calculated from 2 positive samples only.
 ++ Majority of samples recorded as "TNTC= too numerous to count" in samples weighing 50.17 g. Average of 11 "light infection" measured samples = 2565.
 + Twenty samples were not marked as to fish age (14 adults + 27 Jacks + 20 unmarked=61).

Figure 1. Average metacercaria / gram values for Spring-run (SC) and Fall-run (FC) broodstock sampled at Trinity R. Hatchery in 1993, 1994, and 1996.



No correlation of fish age to severity of infection was detected in the most complete data set available (1994 Fall-run, Mann-Whitney rank sum, $p= 0.830$). Numerous encysted metacercariae were observed in the 25 histological kidney specimens collected from Fall-run adults in 1993, however, no inflammation or lesions was associated with the parasites. The majority of the kidney's nephron system of blood vessels, glomeruli, and tubules appeared unaffected by the parasites. In approximately 20 % of the fish, myxozoan parasites were observed in the glomeruli and distal tubules of the kidney. Similar myxozoan parasites are also observed in the kidneys of out-migrant juveniles in the Trinity and Klamath Rivers, however, these infections tend to produce inflammation of the glomerulus (glomerulonephritis). As no mature spores have been seen, the taxonomic identity of the pre-sporogonic forms is unclear. The myxozoans *Chloromyxum*, *Myxidium*, and *Sphaerospora* can be found in the kidney of Pacific salmonids.

Metacercariae were only seen in only 1 of the 46 Spring-run adult samples collected in the Klamath estuary and 1 of the ocean-caught fish. In both cases, only one parasite was observed in the kidney preparation. This data suggests that the majority of metacercariae infect the fish during the up-river migration and pre-spawn holding phase. It is to the parasite's advantage to infect the adult spawner as this lifestage has a higher probability of being eaten by the trematode's final host (fish eating mammal or bird). The 1000-2000 fold increase in body mass from smolt to adult chinook would act to "bio-dilute" the metacercariae acquired as juveniles. Even if the juvenile had a heavy infection of 100 metacercariae in its kidney (avg kidney wt = 0.015 g, $100/0.015= 6667$ metacercariae per gram), the density of metacercariae could drop 1000 fold to 7 metacercariae per gram in a 3 - 4 year adult. This "biodilution" would act to reduce the detection ability from subsamples of the kidney. The lack of morbidity observed in the sample broodstock at TRH suggests that adult chinook can withstand heavy metacercarial infections while in freshwater and still survive to spawn.

R. salmoninarum - The majority (83 - 100%) of all adult kidneys tested had either low RS antigen concentrations or were below the negative cutoff (Fig. 2). The 1992 Spring-run samples, particularly the 3 - 5 yr old adults, had both the greatest number of "positive" level kidneys (17%) and the highest mean O.D. value (0.115) of all the groups (Table 2). In contrast, the ocean / estuary adults captured in 1996 had the lowest O.D. values of the examined groups. Spring-run fish had significantly higher O.D. values than Fall-run fish in 1993 and 1996 (Mann-Whitney Rank sum test, $p= 0.001$). In 1994, the opposite occurred with Fall-run fish with having significantly higher O.D. values (Mann-Whitney Rank sum test, $p= 0.0276$). As seen in metacercariae infection, jacks tended to be similar to adult chinook in their RS infection profile. When age classes were compared within the same year (1992 - 94) and stock (both Spring and Fall-run), only the 1992 spring-run adults had significantly ($p<0.001$) higher O.D. values than jacks (Table 3). It appears that Bacterial Kidney Disease (BKD), as evident by kidney lesions and high RS antigen values, was not prevalent among both spawning broodstock or the ocean / estuary captured fish.

The low-level RS infection observed in most out-migrant chinook juveniles could lead to several scenarios: 1) infection progresses in saltwater to become BKD and a portion of the population dies in the estuary or ocean (Banner et al. 1986), 2) the **RS** infection remains latent in the majority of the fish until the rigors of up-stream migration and spawning result in BKD and some mortality, 3) the low-level RS infection remains as a normal part of the fish's microbial fauna and does not progress into a disease state, or 4) the fish clear their body of this bacterium while in saltwater. The present data cannot make a definitive statement about these scenarios due to various sample biases. The TRH broodstock samples represent fish which have successfully migrated, matured in the river, and were selected by hatchery personnel for spawning. The eight kidney samples from marked, ocean-caught fish were too few in number to make any conclusions about this population, however, both these fish and the 46 estuary samples (beginning upriver migration) had very low-levels of RS antigen (18 of the estuary fish were TRH stock). This ELISA data suggests that Trinity R. adult chinook populations, nearing sexual maturity, did not suffer from severe BKD in 1992-94 or 1996.

Figure 2. Incidence of *R.salmoninarum* antigen concentration in the kidneys of adult Spring-run (S) and Fall-run (F) chinook at TRH or ocean / estuary - caught chinook (OC96). Samples collected in 1992 - 1994 and 1996 were assayed by ELISA and the severity of infection rated by the Optical Density value as **high** (indicative of active infection) or low - to - **moderate** (latent or mild infection).

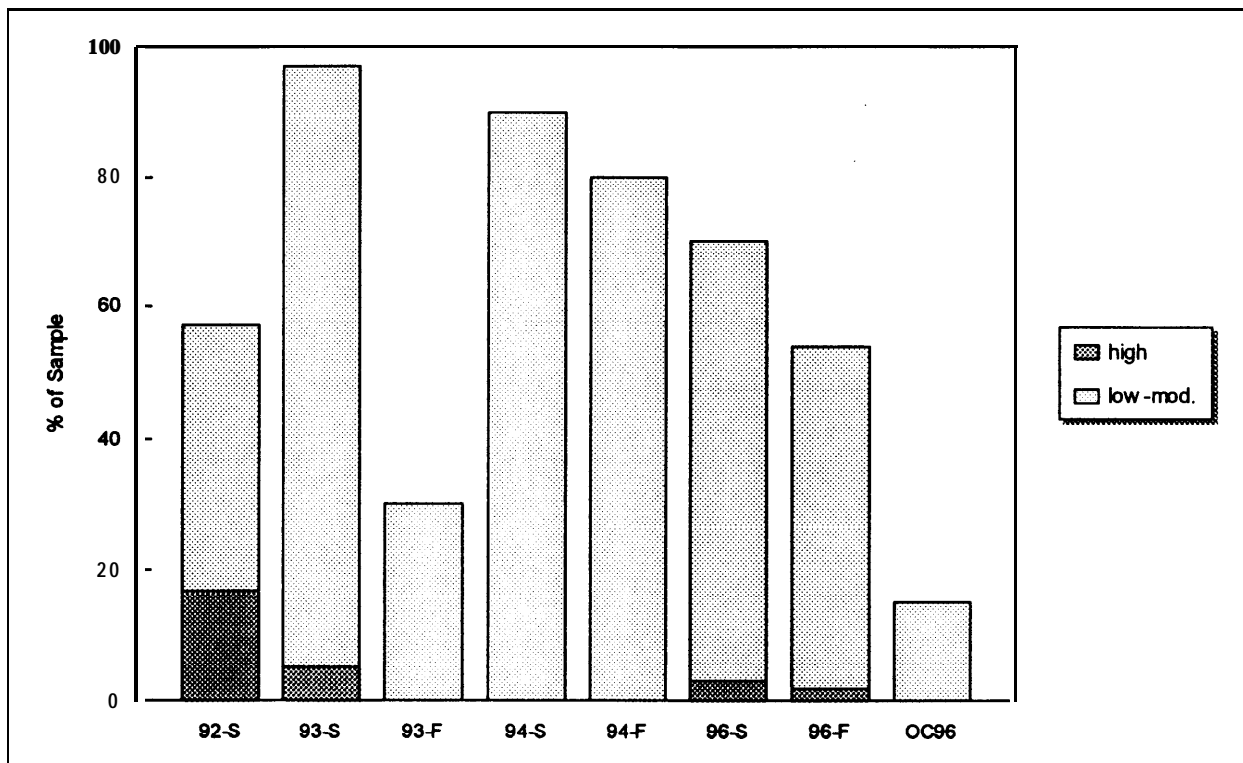


Table 2.

Renibacterium salmoninarum antigen data from Spring-run (SCS) and Fall-run chinook salmon (FCS) used as TRH broodstock (1992-94, 1996) and ocean / estuary adult samples collected in 1996 (96 OCEAN). Categories of infection include Below Negative Cutoff (BNC), low-level or Suspect (SUS), and high or Positive (POS). Infection data reported as number of samples matching a category / total samples collected for that year (%).

	BNC(%)	SUS(%)	POS (%)	OD-Blank Mean (\pm SEM)
92 SCS	26 / 60 (43)	24 / 60 (40)	10/60 (17)	0.115 (.044)
93 SCS	1 / 38 (3)	35 / 38 (92)	2 / 38 (5)	0.035 (.011)
93 FCS	29 / 40 (73)	11 / 40 (27)	0 / 40 (0)	0.009 (.002)
94 SCS	28 / 61 (46)	28 / 61 (46)	5 / 61 (8)	0.070 (.041)
94 FCS	9 / 62 (15)	53 / 62 (85)	0 / 62 (0)	0.014 (.001)
96 SCS	18 / 60 (30)	40 / 60 (67)	2 / 60 (3)	0.030 (.007)
96 FCS	25 / 60 (41)	34 / 60 (57)	1 / 60 (2)	0.008 (.003)
96 OCEAN	48 / 54 (89)	6 / 54 (11)	0 / 54 (0)	0.005 (.001)

Table 3. Age Breakdown of *Renibacterium salmoninarum* antigen data from Spring-run (SCS) and Fall-run chinook salmon (FCS) used as TRH broodstock. Age of the sampled fish was tracked as 2 yr. old male (Jack) or +3 yr. old female or male (Adult). Infection data reported as number of samples matching a category / total samples collected for that year (%) and mean optical density value (\pm standard error of the mean).

	BNC(%)	SUS(%)	POS (%)	OD-Blank Mean (\pm SEM)
92scs Adult Jack	6 / 30 (20) 20 / 30 (67)	14 / 30 (47) 10 / 30 (33)	10 / 30 (33) 0 / 30 (0)	0.216 \pm 0.084 ** 0.140 \pm 0.002
93scs Adult Jack	0 / 19 (0) 1 / 18 (6)	18 / 19 (90) 17 / 18 (94)	1 / 19 (10) 0 / 18 (0)	0.045 \pm 0.022 0.024 \pm 0.004
93FCS Adult Jack	17 / 20 (85) 12 / 20 (60)	3 / 20 (15) 8 / 20 (40)	0 / 20 (0) 0 / 20 (0)	0.007 \pm 0.001 0.011 \pm 0.003
94scs Adult Jack	1 / 15 (7) 2 / 16 (12)	14 / 15 (93) 14 / 16 (88)	0 / 15 (0) 0 / 16 (0)	0.132 \pm 0.098 0.041 \pm 0.023
94FCS Adult Jack	14 / 30 (47) 5 / 32 (16)	16 / 30 (53) 27 / 32 (84)	0 / 30 (0) 0 / 32 (0)	0.012 \pm 0.001 0.016 \pm 0.003

** significant difference by Mann-Whitney test $P < 0.001$.

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