Table 1. Summary of the overall weight of evidence for assessment of AOP 323, following the questions and rankings provided in the AOP Developers' Handbook

	Defining Question	High	Moderate	Low
1. Support for Biological Plausibility of KERs	Is there a mechanistic (i.e., structural or functional) relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding based on extensive previous documentation and broad acceptance -Established mechanistic basis	The KER is plausible based on analogy to accepted biological relationships, but scientific understanding is not completely established.	There is empirical support for a statistical association between KEs (See 3.), but the structural or functional relationship between them is not understood.
MIE => KE1: Activation, PPARα leads to Decreased, cholesterol (KER 2073)	**MODERATE** There is broad understanding of lipid metabolism pathways and empirical evidence (*in vivo* and *in vitro*) that demonstrate the central role of PPARα in lipid and lipoprotein metabolism. Known PPARα agonists (i.e., fibrates) are prescribed to decrease plasma triglyceride and cholesterol levels in humans, with their mode of action involving several mechanisms. While there are intermediate biological steps between these two KEs, this relationship is considered adjacent and there is strong evidence demonstrating that activation of PPARα results in decreased cholesterol. PPARs are conserved across vertebrates and PPARα has been documented in multiple teleost fish along with evidence of cross-species similarity in susceptibility to PPARα agonists including fibrates.			
KE1 => KE2: Decreased, cholesterol leads to Decreased, 11KT (KER 2072)	**HIGH** The process of steroid hormone biosynthesis is well understood, and cholesterol is the precursor for all steroid hormones including testosterone and 11-ketotestosterone.			
KE2 => KE3 Decreased, 11KT leads to Impaired, Spermatogenesis (KER 2076)	**HIGH** 11-ketotestosterone is a dominant androgen in teleost fish and is well documented as necessary for spermatogenesis and sperm production.			
KE3 => AO1 Impaired, Spermatogenesis leads to Decreased, viable offspring (KER 2937)	**MODERATE** The process of spermatogenesis is well understood, and gametes produced from spermatogenesis are required for sexual reproduction. Spermatogenesis and sperm production is necessary to produce viable offspring from sexual reproduction, however other factors are involved in producing viable offspring.			
AO1 => AO2 Decreased, viable offspring leads to Decrease, Population growth rate (KER 2938)	**MODERATE** Population growth rate is dependent on rates of recruitment (instantaneous birth rate) and death (instantaneous death rate). Decreases in viable offspring could therefore lead to decreased population growth rate, recognizing that other factors (e.g., immigration/emigration, juvenile survival, etc.) influence population growth.			

2. Support for Essentiality of KEs	Defining Question	High	Moderate	Low
	What is the impact on downstream KEs and/or the AO if an upstream KE is modified or prevented?	Direct evidence from specifically designed experimental studies illustrating prevention or impact on downstream KEs and/or the AO if upstream KEs are blocked or modified	Indirect evidence that modification of one or more upstream KEs is associated with a corresponding (increase or decrease) in the magnitude or frequency of downstream KEs	No or contradictory experimental evidence of the essentiality of any of the KEs.
AOP	Essentiality of KEs was assessed for the AOP as a whole and is considered **MODERATE** – rationale for individual KEs is included below. - Known PPARα agonists (fibrates) have been used as model chemicals in experimental exposures to demonstrate decreases in total cholesterol with temporal and dose concordance in multiple fish species. This provides some support for essentiality, however, directly measuring agonism of PPARα in fish in vivo studies is challenging and the authors are not aware of any antagonism of blocking or reversibility studies have explicitly investigated this in fish. - The process of steroid hormone biosynthesis is well understood, and cholesterol is the precursor for all steroid hormones, including 11-KT. The relationship between decreased cholesterol and decreased 11-KT is well-established and the essentiality of cholesterol for production of 11-KT is further supported by an *ex vivo* study which showed that exposure to gemfibrozil (a known PPARα agonist) resulted in decreased 11-KT production unless supplemented with 25OH-cholesterol (Fraz et al. 2018), supporting that the decreased steroid synthesis is due to decreased cholesterol availability. - 11-KT is well documented as a critical androgen for proper male reproduction in teleost fish and has well-documented involvement in spermatogenesis and spermiation. The essentiality of 11-KT for spermatogenesis has been documented in zebrafish knock-out studies with rescue (Zhang et al. 2020) which showed that zebrafish with *cyp11c1* knockout have reduced 11-KT levels, smaller genitalia, inability naturally mate, defective Leydig and Sertoli cells, and insufficient spermatogenesis. These effects were corrected by treatment of 100 nM 11-KA (which is converted to 11-KT *in vivo*) for 4 hours per day for 10 days. Thus, demonstrating that spermatogenesis was arrested due to insufficient 11-KT levels. - Spermatogenesis and the production of sufficient quality and quantity of sperm is required for successful oocyte fertilization and production of viable offspring. Exposure studies with known endocrine disruptors (e.g., DEHP, EE2) and knock-out studies targeting genes associated with spermatogenesis (e.g., Tdrd12, AR) and meiotic division (e.g., E2f5, Mettle3, mlh1) demonstrated interference with spermatogenesis (i.e., delayed progression, decrease in sperm volume and motility) and decreased number of fertilized eggs and viable offspring, with dose and temporal concordance. This provides some support for essentiality. - By definition, there must be viable offspring to maintain a population. However, there are other vital rates that are essential here as well, such as survival to reproductive age.			

3. Empirical Support for KERs	Defining Questions	High	Moderate	Low
	Does KEup occur at lower doses and earlier time points than KE down and at the same dose of prototypical stressor, is the incidence of KEup > than that for KEdown? Are there inconsistencies in empirical support across taxa, species and prototypical stressor that don't align with expected pattern for hypothesised AOP?	Multiple studies showing dependent change in both events following exposure to a wide range of specific prototypical stressors. (Extensive evidence for temporal, dose- response and incidence concordance) and no or few critical data gaps or conflicting data	Demonstrated dependent change in both events following exposure to a small number of specific prototypical stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	Limited or no studies reporting dependent change in both events following exposure to a specific prototypical stressor (i.e., endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesised AOP
MIE => KE1: Activation, PPARα leads to Decreased, cholesterol (KER 2073)	**HIGH** There are multiple studies in fish that demonstrate exposure to known PPARα agonists (considered prototypical stressors or model chemicals) resulted in decreased total cholesterol. These studies include experimental exposure of seven different fish species to several different fibrates (clofibrate, Clofibric acid, gemfibrozil, fenofibrate, WY-14643). Temporal and dose concordance was demonstrated in one study (Velasco-Santamaría et al. 2011); however, there is insufficient empirical evidence for development of a quantitative relationship between the KEs. **Uncertainties:** Evidence is based on exposure to known PPARα agonisms, rather than direct measurement of PPARα agonism (which is challenging to measure in fish in vivo studies). There is uncertainty of whether all fibrates shown effective in humans are PPARα agonists in fish; however, a cross-species comparison in vitro and susceptibility evaluation based on gene sequences support similarity in responses across vertebrates.			
KE1 => KE2: Decreased, cholesterol leads to Decreased, 11KT (KER 2072)	**MODERATE** There is strong biological plausibility for this relationship, however, there are relatively few fish studies with stressors that have measured both cholesterol and 11-KT. Two exposure studies that measured both KEs showed dose and			

	temporal concordance, and the third study provided strong evidence of essentiality of cholesterol for the production of 11-KT. **Uncertainties:** Several studies have documented sex differences in one or both of the KEs. In addition, 11-KT levels can be highly variable between fish and have seasonal fluctuations with highest levels at spawning.
KE2 => KE3 Decreased, 11KT leads to Impaired, Spermatogenesis (KER 2076)	**HIGH** There is substantial empirical evidence showing spermatogenesis in fish is dependent on 11-KT, with numerous studies on both higher 11-KT (treatments with 11-KT or increased production) and decreased 11-KT. Several studies demonstrate dose and temporal concordance, with some empirical response-response relationship data that contribute to the quantitative understanding of this KER. **Uncertainties:** There are a few studies that document a significant change in one of the KEs without a significant change in the other. For example, in Hatef, A. et al. (2012), treatment with the anti-androgen vinclozolin at 100 µg/L saw an increase in 11-KT levels with no significant change to spermatogenesis. In this same study, treatment at 400 µg/L saw no significant change in 11-KT levels with a decrease in spermatogenesis.
KE3 => AO1 Impaired, Spermatogenesis leads to Decreased, Viable offspring (KER 2937)	**HIGH** There is substantial empirical evidence demonstrating that impaired spermatogenesis results in decreased oocyte fertilization and a reduction in viable offspring. Much of the cited literature is from fish exposed to prototypical stressors (endocrine disruptors), with several studies demonstrating dose and temporal concordance and a few studies with empirical response-response relationship data that contribute to the quantitative understanding of this KER. **Uncertainties:** Some studies document a significant change in one of the KEs without a significant change in the other. For example, exposure to EE2 did not significantly change spermatogenesis but did result in a decrease in viability of embryos (Schultz et al., 2003).
AO1 => AO2 Decreased, Viable offspring leads to Decrease, Population growth rate (KER 2938)	**LOW** There is limited direct empirical evidence in the literature that population size will decrease with decreased viable offspring. There are no empirical data suitable for evaluating the dose-response, temporal, or incidence concordance between AO1 and AO2. **Uncertainties:** There are multiple factors that influence population growth, including immigration/emigration, intraspecific and interspecific competition, predation, disease.